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MARJORY STEPHENSON

Obituary Notice

MARJORY STEPHENSON, 1885–1948

Marjory Stephenson disliked intensely the rituals connected with illness and death; it is felt that the only tribute to her memory which she would have appreciated would be one which might be of help to others. It is to this end that her contribution to research in chemical microbiology will be described in detail, for it covers almost the whole of the period during which the techniques of modern biochemistry have been applied to micro-organisms. She was one of the pioneers, and her philosophy of research contains much which must be of inspiration to those about to set foot in these fascinating fields.

Marjory Stephenson was born on 24 January 1885 at Burwell, near Cambridge. Except for the period during and immediately before the 1914–18 war she spent the whole of her life in Cambridge and the immediate neighbourhood. At Newnham she read Natural Sciences (Chemistry, Physiology, and Zoology); she always maintained a close link with her College and became a member of the Governing Body in 1931 and of the Council in 1944. After graduating, lack of funds prevented her taking up Medicine as she wished, and the period 1906–11 was spent in the study, and later the teaching, of domestic science at Gloucester County Training College and King's College of Household Science, London. She did not much enjoy this type of teaching and rarely spoke of this phase of her career.

In 1911 came the opportunity (for which she was deeply grateful) to enter biochemical research with R. H. A. Plimmer at University College, London. Here she worked successively on the lactase of intestinal mucosa, the synthesis of palmitic acid esters, and finally on experimental diabetes with E. H. Starling. The tenure of her Beit Memorial Fellowship (awarded in 1913) was however interrupted by the war. She joined the Red Cross and served with distinction in France and at Salonika, being made an Associate of the Royal Red Cross and awarded the M.B.E.

In 1919 she returned to Cambridge to join the enthusiastic group of workers that F. G. Hopkins was gathering, and who, under his leadership, were to make Cambridge the centre of biochemical thought in Britain for a generation. She found this lively environment very much to her taste and indeed helped to make it. Her own admiration and regard for 'Hoppy' is best seen in the obituary notice she wrote in 1948; it was to be unfortunately her last full contribution to this *Journal*. This feeling was reciprocated and her firm common sense was, especially during the 'thirties, a great standby to Hopkins in the running of his department, in which she had by then become a leading personality. With his encouragement she soon turned her attention to micro-organisms, and from then on they were to be the dominating influence in her scientific life. Her genius was for experiment rather than theory, and perhaps this explains their unique fascination for her; in 1930, when considering the place of bacteria in the universe, she wrote in the preface to the first edition of *Bacterial Metabolism*:

> Perhaps bacteria may tentatively be regarded as biochemical experimenters; owing to their relatively small size and rapid growth, variations must arise very much more frequently than in more differentiated forms of life, and they can in addition afford to occupy more precarious positions in natural economy than larger organisms with more exacting requirements.

Her work brought an international reputation, and finally (1945) the signal honour of being (with Dr K. Lonsdale) one of the first two women to be elected to Fellowship of the Royal Society. She was also one of the first women to receive the Sc.D. of Cambridge University, then titular only. It gave her a great deal of fun, when women were finally admitted to full rights of the degree, to observe the reactions of some of the older males when she appeared in her full regalia.

From the time that her Beit Fellowship expired Marjory Stephenson was supported by the Medical Research Council, first by annual grants and after 1929 as a member of their scientific staff. She was highly appreciative of the broad-minded attitude of this body, and served as secretary of their Committee on Chemical Microbiology from its inception until her death. She was an early member of the Biochemical Society (1914) and was a member of the Committee from 1928 to 1932.

Marjory Stephenson always had a deep interest in the biological side of her subject and this broadened as the years passed. She felt indeed that biochemistry owed a debt to biology which was not always recognized. This found expression in her desire that all types of microbiologists should have a closer liaison for their mutual advantage; to this end she was a leading spirit in the formation of the Society for General Microbiology in 1945, and she became its second President in 1947.

Marjory Stephenson (M.S., as she was known to her colleagues) had a vivid and arresting personality;

her feelings—and the expression of them—about people and affairs were always positive. She was intolerant of all forms of pretentiousness, whether scientific or personal. Her independence of judgement did not permit her to adhere consistently to any 'party-line', but her sympathies, and often her active support, were always to be found on the side of progressive movements. She was especially active in the interests of those European scientists whom the fascist regime had forced out of their own countries. In these and many other ways she found an outlet for the personal generosity which was so marked a feature of her human relationships.

She always worked very hard herself and expected the same degree of activity in others. For relaxation she turned, in later life in particular, to her garden, which was a source of great pride and pleasure to her and which she attacked with the same high technical efficiency that characterized her laboratory work. In the last years she became particularly interested in the finer arts of the cultivation of fruit trees.

Marjory Stephenson did not permit the penultimate stage of her illness, with its painful treatment, to affect her scientific activity or her general work for chemical microbiology. She carried on with so much of her usual vigour and essential gaiety that few knew the state of her health. The last stages fortunately passed rapidly; she died on 12 December 1948.

Teaching and direction of research

The teaching of advanced biochemistry in Cambridge is traditionally carried out by the leading workers in various fields whether or not they are members of the University staff. Marjory Stephenson took her full share in this from 1925 onwards. She did not much enjoy formal lecturing; lectures provided the facts, but it was in the informal chat during the practical class that she got in real touch with the student. Her success may be measured by the steady flow of recruits from the Part II Class to her research team.

It was in the guidance of the young research worker that Marjory Stephenson had her greatest influence. Her concern was to see that the novice gained the maximum advantage from his first years of work; she was not interested in an impressive flow of publications from her group. Though always ready with sound and practical advice, she never 'spoon-fed', and was content to allow her young people to test their own mettle, even though she might need to extricate them in the end. She encouraged persistence and insisted on the degree of thoroughness characteristic of her own work. Of Marjory Stephenson it may truly be said that 'infection not instruction is the secret of education'. Visits from the 'great' from other lands were always shared with her research workers, usually by delightful informal entertainment in her own home, where initial shyness or diffidence was so easily dispelled.

She believed firmly that research and teaching were complementary in the sense that each was likely to prove less fruitful unless the other was being actively pursued by the instructor. With the increasing development and interest in chemical microbiology and microbiology in general she worked hard for the establishment of a special Part II Biochemistry (Microbiological) in Cambridge. This was started in 1947, and in the same year the University recognized her long service to teaching by creating her its first Reader in Chemical Microbiology.

Research in chemical microbiology

Marjory Stephenson worked in this field for nearly thirty years (1920–48); during this time she published, either alone or in collaboration, some twenty-two original papers. Each of these was a substantial contribution to the subject; indeed it was characteristic that she did not publish until the work had been thoroughly established and had reached a certain degree of completeness. It is noteworthy that her publications do not include a single 'letter to the Editors'. Another characteristic was that her name never appeared on a paper unless she had been responsible for a full share of the actual work at the bench. It is therefore difficult to assess completely her direct influence in the development of this subject by reference to her own papers alone; much more work published independently by younger members of her team was suggested by her and its successful prosecution made possible by her counsel and aid. The high reputation of Britain in the establishment of this field was largely due to the parallel development of bacterial metabolism under her leadership and that of bacterial nutrition by a team of workers (also supported by the Medical Research Council) under Fildes. As both workers always recognized that they would, these two lines have now converged and the confluence is leading to some of the most important modern developments.

In an address given at the first meeting of the Society for General Microbiology in February, 1945, M.S. defined five levels at which research in microbiology could be undertaken. These were: (1) mixed cultures of organisms growing in natural environments, (2) pure growing cultures in complex media, (3) pure growing cultures in highly purified media (chemically defined), (4) non-proliferating cells in pure culture on chemically defined substrates, and (5) cell-free enzymes and coenzymes on pure substrates. These levels she considered represented only different methods of technical approach and that none should be considered higher or lower than another, although the solution of problems connected with (1) was perhaps the ultimate objective.

The main point was that research should be done concurrently or alternately at different levels, results obtained at one often providing the clue for solving outstanding problems at another. Although she did not formally state this philosophy until almost the end of her research career, it is obvious from her publications, as well as from personal contact, that it had guided her for many years, and had contributed largely to the sense of realism that was so marked a feature of her work. M.S.'s work was mostly at levels (4) and (5), but almost always undertaken as the best method of elucidating some problem connected with the whole growing organism.

When Marjory Stephenson went to F. G. Hopkins's laboratory she joined first in the work on the fat-soluble vitamins (Stephenson & Clark, 1920; Stephenson, 1920). A desire for a deeper understanding of fat metabolism in general led her (with M. D. Whetham) to her first work with bacteria.

> Studies in fat metabolism have hitherto been chiefly carried out on highly specialised vertebrate tissue. By making investigations on a unicellular organism, more susceptible of laboratory control,...

This was probably the only occasion on which bacteria were chosen deliberately as a tool for biochemical research; after that the fascination of the micro-organism itself exerted an ever-increasing grip.

In two papers (Stephenson & Whetham, 1922, 1923) on the fat metabolism of the Timothy grass bacillus (*Mycobacterium phlei*) careful 'balance-sheet' experiments were made with the growing organism. Perhaps the most important observations were, first, that growth could go on even after glucose had been exhausted, and secondly that while acetate alone would not support growth it could be metabolized in the presence of glucose with resultant increase in lipid material. Even in this first work an attempt (unsuccessful) was made to simplify the analysis of a catabolic reaction by cutting out growth. The nitrogen source was omitted from the medium and a heavy inoculum was used; glucose was not, however, attacked under these conditions.

The 'balance-sheet' technique was next applied (Stephenson & Whetham, 1924) to growing cultures of *Escherichia coli*, and the results had a profound influence on her work for many years. With glucose as carbon source no oxygen was taken up for the first 24 hr. even when air was bubbled through the culture, although growth was good and glucose disappeared rapidly. This observation must have focused her attention on the anaerobic way of life, and this was one of her main interests for the rest of her life; it is reflected not only in her own work but also in that of other members of her group. This characteristic aspect of bacterial metabolism intrigued her greatly. Thus she wrote (Stephenson, 1947), in a review on hydrogen transfer:

> Moreover amongst heterotrophs it is as anaerobes that bacteria specially excel...in other words it is in the use of hydrogen acceptors that bacteria are especially developed as compared with animals and plants.

The work quoted above also led her to this field from another point of view; substances such as lactate and succinate would not support the anaerobic growth of *Esch. coli* (though they would do so aerobically), and it was not possible to envisage an exothermic anaerobic breakdown of such substances alone. She now joined forces with Quastel who had been led to similar considerations from his work with *Pseudomonas pyocyanea*. At this period the washed suspension technique (first used by Harden & Zilva, 1915), which was to prove so useful (level 4) for the study of bacterial reactions isolated from the additional complexities of growth, was undergoing rapid development in Cambridge by Quastel and his colleagues mainly with reference to the dehydrogenase enzymes of bacteria. M.S.'s own part in this development was the application of the technique to the problems of anaerobic growth (Quastel, Stephenson & Whetham, 1925; Quastel & Stephenson, 1925). It had been found by the methylene-blue technique that such suspensions activated many substances to act as H donators; it was now found that other substances (e.g. nitrate, fumarate) were activated as H acceptors, i.e. re-oxidized reduced dyestuff. Furthermore, the suspensions brought about oxido-reduction reactions between pairs of such donators and acceptors, e.g.

$$CH_3CHOH.COOH + KNO_3 \rightarrow CH_3CO.COOH + KNO_2 + H_2O.$$

These suggestive findings were immediately tested in growth experiments; under anaerobic conditions growth was usually, though not always, obtained with *Esch. coli* and other organisms, provided that both a donator and an acceptor were present in the medium. Thus while lactate alone would not support anaerobic growth, it would in the presence of either nitrate or fumarate. For growth to occur it was suggested that energy must be liberated by the oxido-reduction reaction, and that a product must be formed capable of entering into the synthetic reactions of the cell.

The next aspect of anaerobic growth tackled concerned the obligate rather than the facultative anaerobes (Quastel & Stephenson, 1926). Here, apart from the question of the nature of the energy-yielding reactions, a second problem presented itself—why was oxygen apparently toxic to the organism? The peroxide theory then current was considered unsatisfactory by the authors whose noses were struck by the fact that *Clostridium sporogenes* always produced sulphydryl compounds

during growth. Following up this hint they demonstrated that cultures or cell suspensions of the organism remained viable even after oxygenation or treatment with hydrogen peroxide (up to about 0·02 %), and grew rapidly on subcultivation into suitable media provided that a source of —SH (such as cysteine) was present; without this there was a prolonged lag. It was suggested that —SH is required to establish a limiting reduction potential essential for the actual growth of these organisms. Perhaps the success which attended this work led to her intolerance with those who complained when a good biological odour was perceptible in the laboratory.

At this stage Marjory Stephenson felt that research on bacterial oxidations with its emphasis on the utilization of dyestuffs, nitrate and so on as oxidizing agents had become in some cases somewhat artificial.

> It is obvious however that for any organisms growing aerobically the most important hydrogen acceptor is molecular oxygen and that consequently a study of aerobic oxidations is essential if a true picture of the normal life of the cell is to be obtained.

This she undertook (Cook & Stephenson, 1928) for the oxidation of glucose and its typical fermentation products, using mainly washed suspensions of *Esch. coli*. Two facts of prime importance emerged. First, such oxidations were found to be largely independent of the viability of the organism; even when this was deliberately reduced to about 0·1 % of the original by ultraviolet irradiation the O_2 uptake was only reduced to a half, and then only initially. Secondly, except in the case of formate, less than theoretical O_2 uptakes were always obtained and no products other than CO_2 could be detected. The explanation for this was not found; it is now known that some carbon is built into cell material (oxidative assimilation).

The last study in this particular phase of her work was her first contribution at level (5) (cell-free enzymes). Following up an observation that the lactic dehydrogenase of suspensions of *Esch. coli* finally increased in activity on storage after a preliminary fall (other dehydrogenases decreased constantly), she succeeded in obtaining a specific cell-free preparation of the enzyme by autolysis of heavy suspensions (Stephenson, 1928). The enzyme could not use O_2 unless a carrier such as methylene blue was present; a search for a source of the missing natural carrier was not successful. This was the first cell-free bacterial enzyme to be obtained, and Marjory Stephenson would certainly have liked to have continued work of this type. Two years later she wrote (in the preface of the first edition of *Bacterial Metabolism*, 1930):

> ...we have indeed much the same position as an observer trying to gain an idea of the life of a household by a careful scrutiny of the persons and material arriving or leaving the house; we keep accurate records of the foods and commodities left at the door and patiently examine the contents of the dust-bin and endeavour to deduce from such data the events occurring within the closed doors.

But the time was not yet ripe; except for the occasional sturdy enzyme which resisted such drastic processes as autolysis, methods were not then available for the extraction of bacterial enzymes. The situation changed in 1938 with the invention by Booth and Green in Cambridge of a wet-crushing mill for bacteria with which it was possible to obtain a number of other enzymes. M.S. and her colleagues at that time (E. F. Gale and J. L. Still) returned to the attack; her own contribution was a full study (Gale & Stephenson, 1939) of the L-malic dehydrogenase of *Esch. coli*. They were not content only to study the kinetics of the enzyme (which required coenzyme I and diaphorase and was reversible); they also demonstrated the presence in *Esch. coli* of substances with the properties and functions of cozymase and diaphorase.

The period 1930–7 represents another phase of Marjory Stephenson's research career. It was perhaps the most fruitful and finally settled her reputation as a world authority in this field. Even if the doors remained locked, her ingenuity of approach permitted her several revealing peeps through the window. It seems particularly appropriate that almost all her own work during this time, as well as much of that of her team, had its origin either directly or indirectly, from a field observation made in her own well loved fenland.

> The culture used by us was originally obtained from the River Ouse, which had been recently subjected to an influx of fermentable carbohydrate material from a beet sugar factory and has given a visible fermentation with evolution of gas in the river itself.

The mud proved a fruitful source of interesting organisms. A mixed culture derived from it reduced sulphate to sulphide and produced methane from formic acid as well as from H_2 and CO_2 (Stephenson & Stickland, 1931*a*). The culture would grow on inorganic salts plus formate; during the first unsuccessful attempts to isolate the methane-producer by enrichment culture, a coliform organism (possibly *Escherichia formica*) was obtained which had the property, in washed suspension, of reducing methylene blue in the presence of H_2. The enzyme had the properties typical of a dehydrogenase and was named hydrogenase. Once discovered, it proved, surprisingly enough, to have a wide distribution among well-known organisms including *Esch. coli*. In one of the rare excursions into hypothesis M.S. permitted herself, she suggested that the function of hydrogenase might be as an intracellular reducing agent,

since many enzymes in cell-free systems are known to lose activity on becoming oxidized (Stephenson, 1947). But the very next paragraph begins:

> But whatever may be the function of the hydrogen-hydrogenase system in the cell it is a remarkably useful tool in the hands of the bacterial chemist, for by its use bacterial reductions can be studied rapidly and quantitatively by manometric methods and the products of reduction obtained unmixed with the products of oxidation.

This was particularly the case with the other two organisms obtained from the Ouse mud, both of which had hydrogenase. The sulphate-reducer was soon isolated; it was a strict anaerobe, morphologically similar to *Desulphovibrio desulphuricans* but not identical with known strains (Stephenson & Stickland, 1931*b*). It grew on the usual salts plus sulphate with fructose, lactate or formate as carbon source. The details of sulphate reduction were studied manometrically with washed suspensions using H_2 itself as donator.

The methane-producing organism proved exceptionally difficult to isolate in pure culture, even after prolonged enrichment on formate medium. Finally it was obtained by the single-cell technique. The organism (which was never identified) grew well on the usual salts with ammonium as N source and formate as C source. Experiments with washed suspensions showed that the energy-yielding reaction was (Stephenson & Stickland, 1933*a*)

$$4H.COOH = CH_4 + 3CO_2 + 2H_2O + 39 \text{ kg. cal.}$$

In the presence of H_2, methane only was formed; CO_2 was also reduced to methane by H_2. It was therefore likely that formate was first split to H_2 and CO_2, part of the latter then being reduced by H_2; in support of this H_2 was found to be present in the early stages. This organism differed from the methane producers so far studied (mostly in mixed cultures) in that only compounds containing one carbon atom yielded methane; these included also CO, formaldehyde and methanol.

The transfer of attention from the utilization of molecular hydrogen to its formation by bacteria followed naturally from the above work, and M.S. and several members of her team turned to a full investigation by the new methods of the production of H_2 from formic acid, which was then regarded as the key intermediate in H_2 production during fermentation. Stickland (1929) had already found that suspensions of *Esch. coli* grown on agar contained formic dehydrogenase, but did not liberate H_2 from formate until they had been in contact with it for some hours and had indeed begun to grow. About this time Karström had introduced the concept of adaptive enzymes, i.e. enzymes found in the bacterial cell only after growth in the presence of the substrate. In the new work (Stephenson & Stickland, 1932, 1933*b*; Yudkin, 1932; Woods, 1936) the enzyme liberating H_2 and CO_2 from formic acid (formic hydrogenlyase) was shown to be present only in cells grown in the presence of formate or substances probably giving rise to formate; strongly aerobic conditions were also adverse for enzyme formation. Although washed suspensions did not become adapted unless a source of N (broth) was present, strong evidence was obtained that the process was not linked to growth, and resulted from a chemical response to the presence of the substrate rather than to natural selection operating on a few organisms already having the enzyme. It is still a matter of doubt as to whether formic hydrogenlyase is a separate enzyme or whether it results from the linkage of formic dehydrogenase and hydrogenase through some electron carrier:

$$HCOOH \rightleftharpoons 2H + CO_2 + 2\varepsilon; \quad 2H + 2\varepsilon \rightleftharpoons H_2.$$

At the time M.S. favoured the first view, mainly on evidence derived from the distribution studies of the three enzymes; more recent work by others favours the second possibility. In 1947, in the review quoted below, she considered the question still open.

The metabolism of H_2 is a process almost unique to bacteria and its study yielded high dividends. She regarded the hydrogenase and hydrogenlyase systems as complementary (Stephenson, 1947):

> The hydrogenase reaction permits an organism to reduce its substrate without using an organic hydrogen donator whilst the lyase reaction enables it to oxidize its substrate without a hydrogen acceptor.

The work with formic hydrogenlyase had stimulated M.S.'s interest in the mechanism of adaptation, and with J. Yudkin she undertook a re-investigation of the long-known adaptation of *Saccharomyces cerevisiae* to growth with galactose as source of energy (Stephenson & Yudkin, 1936). In this case full production of the galactozymase system was obtained by incubating starved washed cells in galactose and buffer only. There was no significant change in either the total or viable count. Further strong, though not conclusive, evidence that adaptation did not depend on cell division came from the observation that 20–50 % adaptation would still be obtained with suspensions whose viable count had been reduced to 2–5 % by irradation with ultraviolet light. Galactozymase was thus probably another case of 'chemical adaptation'. She next made a similar study with *Esch. coli* (Stephenson & Gale, 1937*a*) but with quite different results. The galactose-fermenting system was largely adaptive, but could not be dissociated from growth.

These experiences with adaptive enzymes made a deep impression on M.S., which was reflected by some changes in her general technique of investigation. Thereafter, nearly every piece of work contained its section on the effect on the age of the culture used.

When it is remembered that during the growth of cultures the medium itself is constantly changing, some constituents disappearing and others appearing, it is easy to see that the enzyme activity of growing and even non-growing cultures in the ferment vats must be changing continuously, reflecting as it were, the changing conditions which they themselves have brought about (Stephenson, 1937).

During the 'thirties a good deal of work on the amino-acid metabolism of bacteria had been carried out by several members of the team with her guidance and encouragement. She now entered this field herself with the immediate objective of bringing the full armament of the newer techniques to bear on the old problem of the nitrogen-sparing action of carbohydrate in bacterial growth, characterized by decreased NH_3 formation. In two studies (Stephenson & Gale, 1937*b*; Gale & Stephenson, 1938) the deamination of glycine, DL-alanine, L-glutamic acid and DL-serine was tested with washed suspensions of *Esch. coli* grown under various conditions. In every case the presence of glucose during growth almost totally suppressed enzyme production, whilst the activity of suspensions already containing the deaminases was affected by glucose to a comparatively minor extent. The work, however, was not confined to this limited objective and other important factors (notably degree of aeration and age of culture) were also studied. Strong evidence was also obtained that the serine deaminase required a coenzyme factor; loss of this factor could be prevented in some cases by minute amounts of adenylic acid, and if loss had occurred at low temperatures, activity could be restored in the presence of phosphate by reducing systems. It seems likely from recent work in the U.S.A. that this coenzyme is a biotin nucleotide derivative.

Meanwhile, Gale (1938) had found adenosine to have coenzyme activity for an aspartase enzyme of *Esch. coli*; these findings in her own field, and the increasing general knowledge of the importance of nucleic acid derivatives as components of coenzymes and growth factors, decided M.S. to undertake a general investigation of their function in bacterial metabolism. In characteristic manner she decided that the first task was to get a detailed knowledge of the catabolism, if any, of such substances as adenine, adenosine and adenylic acid. This she did (Stephenson & Trim, 1938); and evidence was obtained that the breakdown of adenosine was the key reaction in all cases. But this programme was interrupted by World War II and was not resumed until nearly ten years later when she extended it to the degradation of ribonucleic and deoxyribonucleic acids (Stephenson & Moyle, 1949).

During the war years she initiated (Davies & Stephenson, 1941) work on the production of solvents (acetone, butyl alcohol) by *Clostridium acetobutylicum*. It was thought that the more clear-cut analysis possible with the washed-suspension technique (previous studies had all been with the growing organism) should throw more light on the factors influencing production of these economically important substances. It proved exceptionally difficult to obtain active cell suspensions even from vigorously fermenting cultures. Age of culture again proved important and it was necessary to wash and resuspend the cells in a complex solution containing broth or yeast autolysate; even then loss of activity was rapid.

M.S. also took part in some of the co-operative trials of microbiological assay methods for vitamins organized during the war by the Medical Research Council.

During the fermentation of plant juices by lactobacilli acetylcholine is often produced; after the war she made a characteristic attack on this problem (Stephenson & Rowatt, 1947). An organism with this property was isolated from Sauerkraut and provisionally identified as *Lactobacillus plantarum*; a full study was made of its general biochemical behaviour. Synthesis of acetylcholine by washed cells was obtained in a simple system of buffer, glucose and choline, and the kinetics studied. Finally, following Lipmann's work on coenzyme A, it was shown that pantothenate-deficient cells showed much reduced synthesis, which was increased fivefold by addition of pantothenate, so that in bacteria also acetylation of choline requires a pantothenate derivative as coenzyme. This was Marjory Stephenson's last completed work and typified her final philosophy of research; a problem arising at level (1), its confirmation at level (2), and detailed investigation at levels (3) and (4). Level (5) was not reached on this occasion, as some attempts to obtain a cell preparation were unsuccessful.

A striking feature of the research as a whole was the wide variety of technical methods used, both biochemical and microbiological. She never shirked using a new method, however difficult technically, even if it were required only to establish some comparatively minor point or to corroborate some matter for which there was already weighty evidence; this was equally true towards the end of her career when she might well have been forgiven for becoming a little conservative. She took very little for granted; whenever possible the products of the reactions she studied were fully identified by proper chemical methods even if their nature was pretty certain and indicated by considerable indirect evidence. This admirable habit of her own generation of biochemists (by no means so common to-day) she maintained to the end.

Marjory Stephenson was essentially an experimentalist; it is really remarkable how few of her papers contained a section formally labelled 'Discussion'; when such did occur it was devoted

rather to practical matters, or to the relation of the work to other fields, than to hypothesis. She was content to let her experimental results speak for themselves, and to lead herself and others to more experiments.

Great as was her regard for bacteria, she did not regard them as creatures of purpose. She considered teleology of no help to the bacterial chemist, although willing to admit that other types of biochemist might be more fortunate; in 1937 she wrote,

> It seems now clear that a belief in the functional importance of all enzymes found in bacteria is possible only to those richly endowed with Faith.

Outside original papers her writing energies were devoted mainly to her book *Bacterial Metabolism*. She wrote comparatively few reviews and these were largely factual; among them were the articles concerning bacteria in the first four volumes of the (then) newly established *Annual Reviews of Biochemistry*. Her book has been the standard work on the subject since its first publication in 1930. Originally conceived as a monograph covering the whole field in detail, the very rapid advance of the subject made it necessary for her to regard the succeeding editions rather as advanced text-books. The lucid style, customary thoroughness, and obvious enthusiasm for the subject made them more than this. Some aspects of the history of science had a strong interest for her, and she had avowed her intention of writing lives of Pasteur and of Hopkins; unfortunately this was not to be.

The most recent developments of chemical microbiology appeared to Marjory Stephenson to bring the subject to its most important and exciting stage, not only for microbiology (in its broadest sense) but for biochemistry also. She had long believed that chemical microbiology had a contribution to make to biochemistry which might come from no other source. This was now happening. Referring *inter alia* to work on the metabolic function of growth factors, enzymic adaptation and biochemical mutants of micro-organisms, she wrote (*Bacterial Metabolism*, 3rd ed.):

> ...such studies are peculiar to microbiology though certainly of wider application; they owe their success to the use of biological material which is prone to biochemical variation and tolerant of interference with its normal biochemical habit.

She was particularly excited about the hopeful beginning in the analysis of anabolic reactions which was being made possible by the use of the microbe as experimental material. In this new phase of research we shall not have Marjory Stephenson with us to play an active part; but the work will certainly go forward the more surely for all that she has done to bring chemical microbiology to its present stage.

Grateful acknowledgement is made to the many friends of M.S. (in particular Dr M. Robertson, Dr D. M. Needham and Dr L. H. Stickland) who have provided information or given me their views on this notice.

D. D. WOODS

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The Elastolytic Activity of Pancreatic Extracts

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According to Lowry, Rourke & Katersky (1941) the insoluble proteins which build up the wall of arteries consist of two scleroproteins, the collagen and the elastin. Of these the elastin cannot be dissolved by any inorganic or organic solvent; the collagen can be dissolved in 2 % acetic acid and when boiled with water it is converted into gelatin. In a previous paper Baló & Banga (1949) showed that histological sections prepared from elastic arteries are dissolved by pancreatic extracts, and examination of the sections stained with resorcinol-fuchsin showed that the elastic fibres building up the wall of the arteries first swell, then break up into particles and finally are entirely dissolved. The dissolving effect of the pancreatic extracts may be explained by the presence of an enzyme, which has been called elastolytic enzyme or elastase.

Work with histological sections requires time, and is not quantitative; it does not involve the isolation of the elastolytic enzyme. Since our investigations showed that dried aorta powder is dissolved by the elastase, as are histological sections, quantitative measurement of the enzyme became possible. In this paper we describe properties of the elastase and methods for the quantitative measurement of its activity.

In connexion with the dissolution of elastin by pancreatic extract the question arises whether this effect may be attributed to the trypsin or chymotrypsin contained in the extract or whether it is the result of the action of a specific enzyme. The literature contains contradictory statements as to whether trypsin dissolves elastin and collagen. There are no adequate data in the literature concerning the enzymic decomposition of the elastic fibres of human and animal arteries. Grassmann (1936) stated that collagen is not digested by trypsin. Ewald (1890) reported that trypsin digests the elastic fibres of the ligamentum nuchae of cattle; in these experiments a pancreatic extract, which might have contained trypsin as well as elastase, was used. Yet Schneider (1937) denied that proteolytic enzymes attack elastin. Thus little definite information exists on the enzymic decomposition of the wall of the arteries.

EXPERIMENTAL

Measurement of elastase activity. For measuring the activity of pancreatic extracts a gravimetric method was used, similar to that employed by Willstätter, Grassmann & Ambros (1926) for measuring the activity of papain on fibrin. These authors weighed the fibrin which remained undissolved after the action of papain. Our method is based on the observation that ethanol-dried aorta powder cannot be dissolved at neutral or slightly alkaline reaction. On treatment with elastase the elastin of aorta powder is dissolved, and the amount dissolved can be determined by weighing the dried matter before and after the action of elastase.

This estimation was done as follows: equal quantities (20 mg.) of aorta powder were put into weighed centrifuge tubes of 6 ml. capacity and into each tube 1 ml. of carbonate-HCl buffer of pH 10·32 was added. To the experimental tubes different quantities of elastase or pancreatic extract were added; no enzyme was added to the control tubes. The volume in each tube was made up with water to 2·5 ml. The tubes were incubated at 38° for 30 min. with constant shaking; they were then centrifuged and the supernatant discarded. The loss of weight from the control tubes is caused by the dissolution of the soluble proteins of the aorta wall, whilst the loss of weight from the experimental tubes is due in addition to the elastin which is dissolved by the action of the elastase. After centrifugation the sediment was washed with 5 ml. of 96 % ethanol and after drying at 110° the tube and its contents were weighed. The difference between the weights of dry substance in the control and in the experimental tubes gives the weight of the elastin dissolved.

Formaldehyde titration. Formaldehyde titration according to Sørensen was used for the quantitative estimation of trypsin activity with casein as substrate. We also examined the activity of our pancreatic extracts containing the elastolytic enzyme upon casein and upon aorta using formaldehyde titration.

Estimation of proteins. In estimating the activity of elastolytic enzyme it is necessary to know the protein contents of the pancreatic fractions. This was done as follows: 5 ml. of acidified enzyme solution containing 1 % (w/v) NaCl were added to weighed centrifuge tubes. The solutions were then heated for 30 min. at 100°. The coagulated protein was centrifuged off and washed twice with 5 ml. of 96 % ethanol. The tubes were dried at 110° and weighed.

Calculation of elastolytic activity. We took as basis the elastolytic units (E.U.) defined in a previous paper (Baló & Banga, 1949): 1 ml. of 1 : 1000 diluted saline extract of

powdered pancreas contains 1 E.U. This concentration can dissolve, in 12 hr. at 37°, histological sections of a thickness of 20 μ. prepared from elastic arteries. This unit is equal to the quantity of enzyme which dissolves, under the conditions used in our present experiments, 5% of the elastin. We determined the E.U. values and the quantity of protein in each fraction used. The activity is equal to E.U./mg. of protein.

Preparation of pancreatic extract. Defatted and powdered pancreas (1 g.) was extracted with 20 ml. of 0·1 N-phosphate buffer (pH 6·0) by shaking it for 30 min. The solution was centrifuged and the precipitate suspended again in 20 ml. of 0·1 N-phosphate buffer (pH 6·0), again shaken for 30 min. and centrifuged. The two solutions were combined and the activity determined. The mixture (1 ml.) contained 25 E.U. and 6·75 mg. of protein. The activity therefore was 25/6·75 = 3·7.

Purified elastase. To 30 ml. of the pancreatic extract described above $(NH_4)_2SO_4$ was added to 0·2 saturation and kept at 2° for 12 hr. The precipitate was centrifuged and discarded. To the solution $(NH_4)_2SO_4$ was added again to 0·4 saturation, and the precipitate, which contained the active elastase, was suspended in 15 ml. of water. The solution was dialysed until free of sulphate and centrifuged. The solution, to which NaCl was added to a final concentration of 1%, was kept at 0°. This solution contained 50 E.U./ml. and 1·55 mg. of protein/ml. (activity = 50/1·55 = 32·1). The elastase prepared in this way was 8·5 times as active as the original pancreatic extract.

Trypsin. Trypsin and chymotrypsin were prepared from fresh pig pancreas by the method of Kunitz & Northrop (1935–6). The fraction, which precipitated with $(NH_4)_2SO_4$ between 0·4 and 0·7 saturation, was used as trypsin, although it contained chymotrypsin as well. The trypsin solution so obtained contained 5 E.U./ml. and 5·4 mg. of protein/ml. (activity, 0·9); it showed very high proteolytic effect on casein.

Preparation of collagen. Human tendo Achillis was cut very finely and ground during 20 min. with 20 vol. of 2% (v/v) acetic acid. It was centrifuged, the fluid decanted and the extraction procedure repeated on the insoluble residue. The supernatant fluids which contained the collagen were mixed and the protein content determined. On adjusting the pH of the acetic acid-collagen solution to pH 8·0, the collagen precipitated in a loose form. The collagen was washed first with water and then with ethanol, and dried in a desiccator.

Preparation of elastin from the aorta wall. The purified aorta wall was ground very finely for 20 min. with 5 vol. of 2% (v/v) acetic acid and then centrifuged; the insoluble material was similarly ground with acetic acid and centrifuged again. The insoluble material was extracted twice with water, washed with 96% ethanol and dried in a desiccator.

RESULTS

The effect of concentration of pancreatic extract on the dissolution of aorta powder

Fig. 1 shows how the dissolution of aorta powder proceeded in the presence of different quantities of pancreatic extract. With increasing quantities of enzyme the dissolution of aorta powder also increased.

Effect of time on the action of elastase

The velocity with which the pancreatic extract dissolves the elastin of aorta was examined. As Fig. 2 shows, the rate of dissolution is greatest at the beginning. During the first 30 min. of incubation 50% of the aorta protein was dissolved and the maximum value was reached after 180 min. At this time 75–80% of the aorta protein was decomposed.

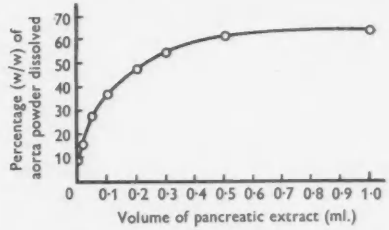

Fig. 1. Dissolution of aorta powder in the presence of increasing quantities of pancreatic extract after incubation for 30 min. at 37°. Total volume 2·5 ml.; 20 mg. of aorta powder; 0·5 ml. of carbonate-HCl buffer, pH 10·32.

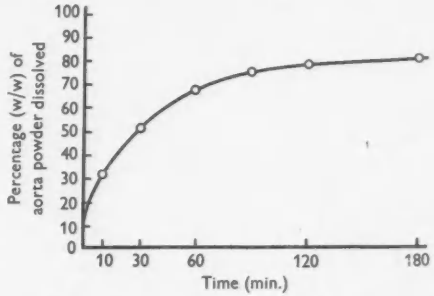

Fig. 2. Effect of time on the dissolution of aorta powder by pancreatic extract (expressed as percentage); 5 E.U. to 20 mg. of aorta powder in 2·5 ml. total volume, pH 10·32; temp., 38°.

There was no further dissolution during longer incubation. This proves that there is a protein fraction amounting to 20–25% which remains unaffected by the pancreatic extract, which contained trypsin as well as elastase. As our further experiments show, this protein is probably collagen.

pH-*activity curve of elastase*

The pH optimum of elastolytic activity was determined by dissolving aorta powder in presence of elastase at different pH's. In order to measure the initial velocity of the reactions a 10 min. incubation was chosen. Aorta powder (10 mg.), buffer (0·5 ml.) and 6 E.U. were used in a total volume of 2·5 ml.

Between pH 3·0 and 6·0 acetic acid-sodium acetate, between pH 5·0 and 8·0 phosphate, between pH 7·0 and 9·0 borate and between pH 9·0 and 11·0 sodium carbonate-hydrochloric acid buffers were used. There

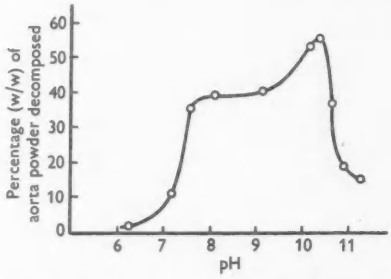

Fig. 3. Decomposition of aorta powder at different pH in the presence of elastase. In 2·5 ml. total volume, 10 mg. of aorta powder, 0·5 ml. of buffers of different pH and 6 E.U. Time of incubation, 10 min.

is practically no enzymic action below pH 6·0. Fig. 3 shows that above pH 6 the activity of elastase increases with alkalinity and that the optimum is at pH 10·3.

The hydrolytic effect of trypsin on aorta and casein

In this experiment the hydrolytic effect of trypsin on the aorta wall was compared with the effect of casein. For following the rate of hydrolysis the Sørensen formaldehyde titration was used. Fig. 4

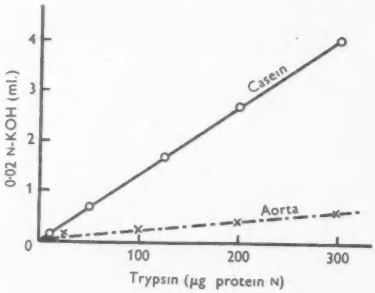

Fig 4. Proteolytic effect of trypsin on casein and aorta. In 2·5 ml. total volume, 20 mg. of casein and aorta respectively, 0·5 ml. of phosphate buffer (pH 8) and different quantities of trypsin. After 20 min. incubation the proteolytic activity of trypsin was estimated by formaldehyde titration.

shows the action of different quantities of trypsin upon casein and aorta. Trypsin liberates very small quantities of amino-acids from the aorta wall in conditions in which the hydrolysis of casein was very marked. This experiment proves that the aorta wall is not appreciably hydrolysed by trypsin. Neither can the liberation of amino-acids be demonstrated following the action of elastase. The purified elastase contains traces of trypsin so the above experiment proves that during the enzymic dissolution of the aorta no amino-acids are liberated. The effect of elastase is only to transform the insoluble protein of aorta into a soluble one.

The effect of elastase on elastin and collagen

It was interesting to know whether in the aortic wall collagen, elastin or both are attacked by elastase. We investigated this question as follows: 10 mg. of elastin and the same quantity of collagen were placed in weighed centrifuge tubes. The test tubes contained 5 E.U. while the control tubes contained no elastase. After 30 min. incubation, 6 ml. of 96% ethanol were added to each tube and the tubes were centrifuged. The precipitates were washed once with ethanol, and dried at 110° to constant weight. In these conditions 67% of the elastin was dissolved by the elastase, but of the collagen only a negligible proportion (about 1%) was dissolved. This experiment proves that in the aorta wall the elastin is the protein which is decomposed by the elastolytic enzyme.

DISCUSSION

The enzymic dissolution of the aorta wall is an important phenomenon from the point of view of arteriosclerosis. The pancreatic extract contains several proteolytic enzymes. Among these trypsin and chymotrypsin are the best known. The elastolytic effect of the pancreatic extract is, we believe, due to a specific enzyme, which dissolves the elastic fibres. Our experiments prove clearly that on removal of the collagen from the aorta, by repeated extraction with 2% acetic acid, the remaining elastin can be dissolved by the elastase. On the other hand, the collagen prepared from the tendo Achillis cannot be dissolved by the elastolytic enzyme. It is evident that in the aorta the elastase dissolves the elastin, but not the collagen.

Experiments dealing with the relation of the elastolytic enzyme to trypsin seem to prove that trypsin is not responsible for the dissolution of the aorta. Pure trypsin and chymotrypsin, which have strong proteolytic activity, showed a much smaller elastolytic effect than the pancreatic extract or the purified elastase. This is a proof of the assumption that elastase is not identical with trypsin or chymotrypsin. Further evidence is provided by the fact that the decomposition of the aorta wall takes place by a mechanism different from that of ordinary enzymic hydrolysis of other proteins. With elastase no amino-acids are liberated from the proteins of the aorta wall, though its elastic fibres are entirely dissolved. Therefore the elastolytic enzyme does not

split peptide linkages, but acts in rendering the elastin soluble. It is our belief that possibly the long rod-shaped molecules of elastin are transformed into globular ones without the liberation of amino-acids. Verification of this hypothesis requires further investigation.

The occurrence of an elastolytic enzyme in the human or animal organism has not been shown before. Eijkman (1904) studied the elastin-dissolving activity of bacteria. He prepared, from the lungs of calves, from ligamenta nuchae and from arteries, culture media with which he demonstrated the lysis of elastin as the result of bacterial action. *Bacillus pyocyaneus* showed the highest activity.

SUMMARY

1. Pancreatic extract contains an enzyme which dissolves the elastic fibres of the aorta wall and which is called elastolytic enzyme or elastase.

2. Trypsin prepared according to Kunitz & Northrop (1935–6) has little effect on arteries.

3. The elastase dissolves only the elastin in the aorta wall and leaves the collagen unchanged.

4. Elastase does not liberate amino-acids from the aorta wall, but insoluble elastin is changed into a soluble protein.

5. The pH optimum of the elastase (10·3) is more alkaline than that of trypsin.

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The Reactions of Catalase in the Presence of the Notatin System

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If hydrogen peroxide (often termed 'bottle hydrogen peroxide' in order to distinguish it from that generated continuously by an oxidizing system) is added to a catalase solution which is concentrated enough for spectroscopic measurements the hydrogen peroxide is all decomposed into oxygen and water in a fraction of a second, thus leaving no time to observe any change in the absorption spectrum of the mixture by ordinary methods. However, by applying the rapid spectrophotometric technique to this reaction it has been possible to detect and to record the absorption spectrum of the catalase-hydrogen peroxide complex which will be referred to as the primary complex (Chance, 1947*a*). If hydrogen peroxide is continuously generated by an oxidizing system, catalase is maintained saturated with hydrogen peroxide for several minutes, and the spectrum of catalase-hydrogen peroxide can be studied by an ordinary spectrophotometric method.

Under these conditions, it has been found that the primary catalase-hydrogen peroxide complex was converted into a new catalase-hydrogen peroxide complex, and that the persistent absorption spectrum which was thus observed belongs to a secondary complex. This complex is catalytically inactive and is analogous to the secondary complex formed by catalase in the presence of alkyl hydrogen peroxides (Chance, 1949*d*). The secondary complex which is also formed in the presence of a solution of 'bottle hydrogen peroxide', is the principal cause of the inactivation of catalase which takes place during the determination of its activity, 'Katalasefähigkeit' (Kat. f.).

If an alcohol and catalase are added to a system composed of an enzyme, catalysing the oxidation of its substrate by means of molecular oxygen which is reduced to hydrogen peroxide, the catalase, instead of decomposing hydrogen peroxide to molecular oxygen and water, uses the peroxide for the oxidation of the alcohol to aldehyde (Keilin & Hartree, 1936). Thus hydrogen peroxide, which is continuously generated when glucose oxidase (or notatin) catalyses the oxidation of glucose to gluconic acid, can be used by catalase for the coupled oxidation of alcohols. A remarkable feature of this reaction is that all the hydrogen peroxide formed in the primary

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oxidation reaction is used by catalase in the coupled or peroxidatic oxidation of the alcohol and none is decomposed catalytically to oxygen and water (Keilin & Hartree, 1945).

It has already been shown that the primary catalase-hydrogen peroxide complex reacts with alcohols (Chance, 1947*a*) and causes their oxidation: but the catalase-hydrogen peroxide complex also participates in the decomposition of hydrogen peroxide into water and oxygen. The latter reaction is shown here to be suppressed in coupled oxidations because of the extremely low steady-state concentration of hydrogen peroxide (approx. 10^{-9}M).

The rapid reaction between the primary catalase-alkyl hydrogen peroxide complexes and hydrogen peroxide (Chance, 1949*c*) has been further studied in the presence of the notatin system, and the turnover of a large amount of catalase-bound alkyl hydrogen peroxide has been measured quantitatively. In this reaction, hydrogen peroxide reacts about 30,000 times as rapidly as ethanol.

METHODS

Preparations. The notatin preparation was dissolved in water and, after centrifuging off some insoluble material, was found to have essentially the same absorption spectrum as the pure material; the ratio of extinctions at 380 and 450 mμ. is 1·16, while the ratio for the pure material is given as 1·04 (Keilin & Hartree, 1948). The concentration was determined by measuring the extinction of the solution at a wavelength of 450 mμ. From this the millimolecular extinction coefficient $\epsilon_{\mathrm{mM}}^{450}$ was found to be 11 from the expression

$$\epsilon_{\mathrm{mM}} = \frac{1}{cl} \log \frac{I_0}{I},$$

where c = mM-concentration of notatin (assuming the sample to be pure notatin), l = depth of liquid layer in cm., and I_0 and I = intensities of incident and transmitted light respectively.

One of the horse-liver catalase preparations used here was kindly supplied by Dr E. F. Hartree, the others by Dr R. K. Bonnichsen.

Method. Some of the reactions studied in this paper are relatively rapid and it has been necessary to use a modified form of the flow method of Roughton & Millikan (1936). In order to conserve material, the speed of response and the sensitivity of the photoelectric spectrophotometer have been increased several 100-fold over that used by Millikan (1936). Thus the extinction of the solutions may be recorded in about 0·01 sec., and thereby the duration of the flow of liquid through the apparatus may be very brief and the total expenditure of reactants is proportionately reduced. The greatly increased sensitivity of the apparatus permits the use of very dilute enzyme solutions (approx. 1×10^{-6}M-haematin iron in a 1 mm. bore capillary observation tube) and further contributes to the fluid economy. A great practical advantage of using such dilute solutions is that the halftimes of second-order reactions, which are usually found in enzyme systems, are proportionately longer in these dilute solutions and the measurement of extremely short time intervals (< 3 msec.) is unnecessary. In fact some of the reactions described in this paper fall in the time range above 50 msec., and can be measured spectrophotometrically after the flow of reactants through the observation tube has been abruptly stopped. In this case the fluid economy is much higher than in the flow method.

In a current model of the apparatus shown in Fig. 1 two 1 ml. tuberculin-type glass syringes (*A*) are fused into a stopcock (*B*) to permit their filling with the two reactants to be mixed, or their manual discharge into a four-jet (0·4 mm. bore) mixing chamber (*C*) and 1 mm. bore observation tube (*D*) which are fused to the barrel of the stopcock. Optical measurements are made usually between 8 and 13 mm. beyond the point of mixing. Mixing is complete in less than 1 msec. with this type of apparatus.

Light from the exit slit of a double-grating monochromator (*E*) illuminates the observation tube and the light transmission of the reactants is measured by an RCA-type 929 photocell (*F*). The wavelength range is 370–630 mμ., although the most effective range for the Soret bands of haematins is 370–450 mμ.

Rapid recording and high sensitivity of the optical measurement are obtained only by accurate stabilization of the intensity of the incident light from the light source (*M*) and by careful design of the electronic circuits (*L*) for such stabilization and for the photoelectric measurement of the transmitted light. Currently available circuits measure extinctions with an error of $\log I_0/I \approx 10^{-4}$. Thus it is possible to record the spectroscopic shift at 405 mμ. from catalase to the primary catalase alkyl hydrogen peroxide complex in solutions as dilute as 10^{-7}M (4×10^{-7}M-haematin iron), but such high sensitivity is rarely necessary.

The design principles of such circuits are well known, and their fundamental limitations of the performance can be reasonably well specified mathematically (Chance, 1947*b*). The output of the amplifying circuits is recorded automatically by pen and moving paper for slow reactions (*G*, Fig. 1) or by mirror galvanometer (*H*) and moving photographic paper for rapid reactions (see Figs. 12–14).

Since the flow velocity is not maintained constant, but is manually raised rapidly from zero to some maximum value and then decreased to zero again in order to conserve enzyme solution, the instantaneous values of the flow velocity are measured and are recorded photographically by a second mirror galvanometer on the same record as the optical density trace. A convenient method for measuring flow velocity electrically is to connect the syringe plungers to a potentiometer (*I*) so that a voltage (or current) proportional to displacement is measured. Such a current is readily differentiated to give flow velocity by various electrical circuits, for example, by a series-connected resistance and capacitance (*J*). The output of the latter circuit is amplified (*K*) and recorded (*H*).

The actual value of time after mixing is computed from the quotient of a constant obtained in a separate calibration involving the various mechanical, electrical and hydraulic factors of the apparatus and the magnitude of the oscillograph deflexion.

A great advantage of recording both the flow velocity and extinction traces on moving film (*H*) is that both 'flow' and 'stop' data are recorded, and the single record covers a time range from about 3 msec. to 10 or more sec.; and as much longer as is desired on the ink recorder (*G*). There is, however, a gap in the data from the stop and flow methods between about 10 and 50 msec., but this causes no practical difficulties in these studies.

In certain experiments it is desirable to record the concentration of O_2 or hydrogen peroxide in the observation tube. Spectrophotometric measurements at these wavelengths are not practicable and a polarographic method diffusion current may be measured as shown by Davies & Brink (1942). For hydrogen peroxide, the electrode is connected to a positive potential (approx. 0·4 V.) (Chance, unpublished data; Bonnichsen, Chance & Theorell, 1947). The

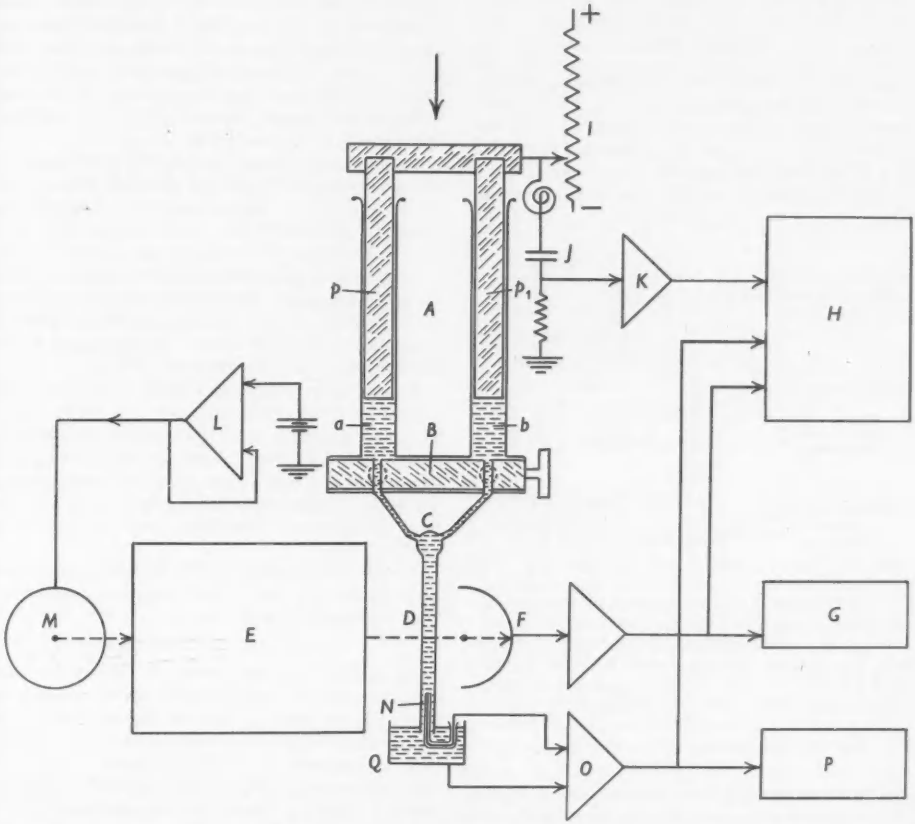


Fig. 1. Schematic diagram of apparatus for the 'accelerated' and 'stopped' flow methods. *A*, two 1 ml. tuberculin-type glass syringes. Plungers *p* and p_1 are connected at the top by a bar and are discharged manually by pushing on this bar. 0·2 ml. suffices for a complete record. *B*, two-way stopcock for filling syringes with reactants or for discharging reactants into mixing chamber and observation tube. *C*, four-jet mixing chamber (0·4 mm. jets). *D*, bore observation tube (1·0 mm.). *E*, double grating monochromator, 370–1000 mμ. (Coleman Electric Co.). *F*, RCA-type 929 photocell and high gain amplifier. Gain approx. 10^8, band width 0–0·1 or 100 cyc./sec., noise approx. 2 μV. *G*, *P*, pen and ink recorders, 0–0·5 cyc./sec. (Esterline-Angus Co.). *H*, three-channel galvanometer oscillograph and moving paper camera, 0–750 cyc./sec. (General Electric Co.). *I*, displacement-measuring potentiometer; linearity 0·2%. *J*, resistance-capacitance differentiating circuit connected by a flexible wire to displacement potentiometer. *K*, flow-velocity amplifier, 0–100 cyc./sec., gain 100. *L*, lamp voltage-control amplifier; stabilization ratio approx. 7×10^4 amp./V. *M*, tungsten lamp (8·5 V., 4·0 amp.). *N*, platinum micro-electrode, approx. 0·1 mm. wire (see detail). *O*, amplifier for micro-electrode including polarizing circuit, 0–10 cyc./sec., gain approx. 10^5, noise approx. 2 μV. *Q*, reference electrode; here a brass cup is used.

based on the platinum micro-electrode is employed, since the electrode is small enough to be inserted directly into the capillary observation tube. For O_2 determinations, the electrode (*N*) is connected to a negative potential and the reference electrode (*Q*) may be calomel, although a brass cup has often been used. The current is amplified (*O*) and recorded on a separate trace of the galvanometer oscillograph (*H*) or on a separate pen and ink recorder (*P*). This amplifier

contains special circuits which reduce the effect of flow velocity through the observation tube upon the electrode current (Chance, unpublished data). These circuits cause some error in the recording of rapid change of O_2 tension, but give satisfactory results for the studies of this paper.

RESULTS

The general characteristics of the reactions of catalase in the presence of the notatin system. Fig. 2 (column *A*) shows the reaction kinetics of oxygen, notatin, and catalase as measured in the apparatus shown in Fig. 1. The records of catalase kinetics at 405 mμ. and notatin kinetics at 460 mμ. were measured

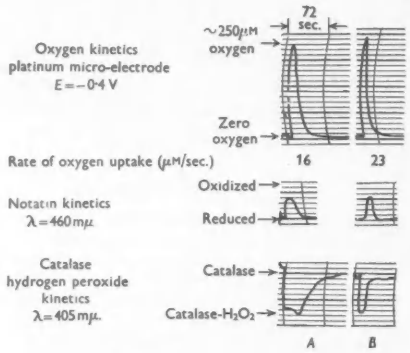


Fig. 2. The kinetics of oxygen, notatin and catalase in the presence of glucose (column *A*) or glucose and ethanol (column *B*); 0·95 μM-notatin, 0·54 μM-horse-blood catalase, 2 mM-glucose, 100 mM-ethanol (column *B* only); pH 6·5, 0·01 M-phosphate. The time interval between the vertical lines in the records is 72 sec. The horizontal lines on the records are not related to the calibration marks at the margins of the records.

spectrophotometrically. At these two wavelengths, there is little mutual interference between the extinction changes caused by the formation of catalase-hydrogen peroxide from catalase or oxidized notatin from reduced notatin. The oxygen kinetics were measured by means of a platinum micro-electrode. In the first column, *A*, the abrupt rise of the top trace indicates the moment at which the deoxygenated solution, remaining in the observation tube of the flow apparatus from the preceding experiment, is flushed out and replaced by a freshly oxygenated solution. At the same time, reduced notatin is replaced by the oxidized form as shown by the rise of the spectrophotometric record at 460 mμ. Since an excess of glucose is present and the observation tube of the flow apparatus is closed to the air, the oxygen uptake of the notatin system begins and causes the decrease of oxygen concentration indicated by the fall of the trace in the platinum micro-electrode record. The oxidase reaction follows this equation:

$$C_6H_{12}O_6 + O_2 + H_2O \rightarrow C_6H_{12}O_7 + H_2O_2. \quad (1)$$

The hydrogen peroxide formed immediately combines with catalase as shown by the spectrophotometric record at 405 mμ. In the identical experiment preceding the one shown, the reaction product is free catalase so that the observation tube is initially filled with free catalase. The abrupt drop of the trace indicates the rapid formation of the catalase-hydrogen peroxide complex.

Contrary to the experiments of the previous paper, where a solution of hydrogen peroxide was added to catalase and the complex began to disappear immediately (Chance, 1947*a*), the continuous production of hydrogen peroxide by the oxidase system (indicated by the continuous fall of oxygen tension in the platinum micro-electrode trace) gives a constant concentration of the catalase-hydrogen peroxide complex for roughly 25 sec., as indicated by the steady deflexion of the trace at 405 mμ.

As the oxygen concentration decreases, more notatin becomes reduced, and the production of hydrogen peroxide from the notatin system finally stops. Catalase breaks down the remaining hydrogen peroxide, and then the catalase-hydrogen peroxide complex spontaneously decomposes into free catalase as described previously (Chance, 1947*a*).

In this experiment, all the hydrogen peroxide is decomposed into water and oxygen by catalase so that the complete equation is

$$2C_6H_{12}O_6 + O_2 \rightarrow 2C_6H_{12}O_7. \quad (2)$$

As Keilin & Hartree (1945) have shown, the addition of an alcohol to this system causes catalase and hydrogen peroxide to oxidize the alcohol to aldehyde instead of decomposing the hydrogen peroxide into oxygen and water. The records of column *B* (Fig. 2) show the effect of 100 mM-ethanol upon this system. First the decrease of oxygen concentration is more rapid since scarcely any oxygen is being supplied from the decomposition of hydrogen peroxide by catalase. Thus the time for the exhaustion of all the oxygen in the capillary observation tube is much less. The catalase-hydrogen peroxide complex forms as before, but in this case the steady-state concentration of the complex is slightly less than the saturation value obtained previously. When the generation of hydrogen peroxide stops due to the exhaustion of the oxygen, the catalase-hydrogen peroxide complex decomposes very rapidly because of its reaction with the ethanol present.

The equation for the reaction occurring when all of the hydrogen peroxide is used in the oxidation of ethanol by catalase is

$$C_6H_{12}O_6 + O_2 + C_2H_5OH \rightarrow C_6H_{12}O_7 + H_2O + CH_3CHO. \quad (3)$$

The Soret bands of the primary and secondary catalase-hydrogen peroxide complexes. The above data show that the catalase-hydrogen peroxide complex is stable when hydrogen peroxide is being generated by the notatin system. If an ordinary spectrophotometer is used, it is necessary to stabilize complex I for several minutes, and therefore the generation of hydrogen peroxide from the dissolved oxygen is prolonged by using more dilute notatin than in Fig. 2. As shown later, ethanol present in the catalase preparation must be removed by a 24 hr. dialysis or the saturation value of complex I may not be obtained.

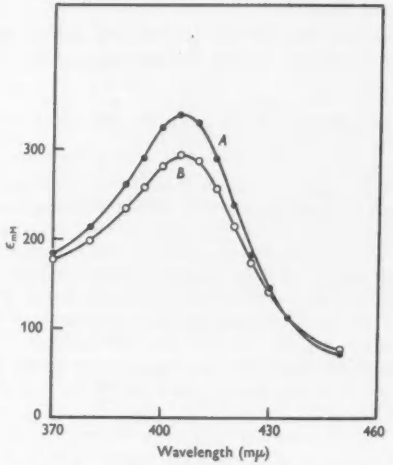


Fig. 3. The Soret band of catalase (*A*) and of catalase-hydrogen peroxide I (*B*) obtained in the presence of notatin, glucose and oxygen; 1·4 μM-horse-liver catalase, $6{\cdot}4 \times 10^{-9}$ M-notatin and 3·3 mM-glucose; pH 6·5, 0·01 M-phosphate.

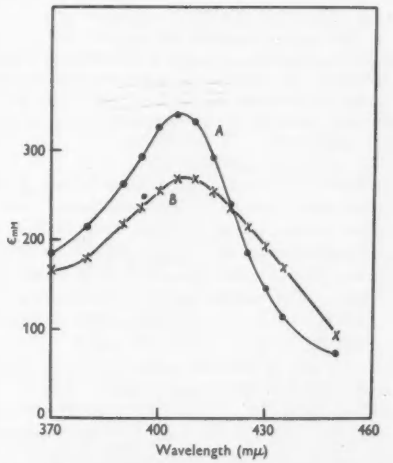


Fig. 4. The Soret band of catalase (*A*) and of catalase-hydrogen peroxide II (*B*) at pH 6·5. The conditions are similar to those of Fig. 3 except that 45 min. elapsed since mixing the solutions. Also the solution was occasionally stirred; 1·3 μM-horse-liver catalase, $1{\cdot}9 \times 10^{-8}$ M-notatin, 6·7 mM-glucose, pH 6·5, 0·01 M-phosphate.

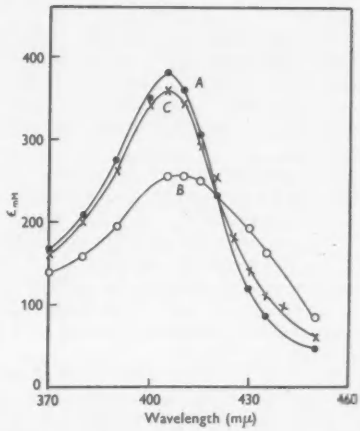


Fig. 5. The Soret band of catalase (*A*) and of catalase-hydrogen peroxide II obtained from horse-erythrocyte catalase (*B*) and the nearly complete recovery of catalase from complex II by stopping hydrogen peroxide generation (*C*); 1 μM-horse-blood catalase, 1×10^{-8} M-notatin, 6 mM-glucose, pH 6·5, 0·01 M-phosphate. Curve *B*, 30 min. after mixing; curve *C*, 1 hr. after covering solution with paraffin oil.

The spectrum of complex I obtained in this way is shown in Fig. 3 (*B*), and is in remarkably good agreement with that obtained by the use of the flow method and a solution of hydrogen peroxide. Here the change of ϵ_{mM}^{405} is 45, and the isobestic point between catalase and complex I is found at 435 mμ. In another experiment, using horse-blood catalase instead of horse-liver catalase, the change of ϵ_{mM}^{405} is 48. Thus the number of catalase haematins bound to peroxide is again shown to be independent of a reduction in the number of intact haematins from 4 to 3 (Chance, 1949*b*).

If a solution of catalase and the notatin system, similar to that used for obtaining Fig. 3, stands exposed to air so that the generation of hydrogen peroxide continues for about 0·5 hr., a very definite change in the spectrum of complex I is obtained as shown in Fig. 4 (*B*); increased absorption appears in the region of 430 mμ. as has been observed upon the formation of the secondary catalase-alkyl hydrogen peroxide compounds. Thus it appears that the

Table 1. *A comparison of the Soret bands of complex II formed in the presence of either the notatin system or ascorbic acid*

(0·91 μM-horse-erythrocyte catalase. Exp. 326.)

Experimental conditions	Wavelength (mμ.) ...	370	380	390	400	405	410	415	420	425	430	435	440	450
4 mM-Glucose, approx. 10^{-8} M-notatin, 0·001 M-phosphate buffer acidified with HCl to give pH 3·8	ϵ_{mM}	134	150	183	225	241	244	233	220	211	196	172	138	76
1 mM-Ascorbic acid, 0·001 M-acetate buffer, pH 4·2	ϵ_{mM}	127	145	174	217	233	239	244	237	220	208	182	149	93

primary catalase-hydrogen peroxide complex has been slowly converted into a secondary and presumably inactive complex. The number of catalase haematins converted into the secondary complex and the kinetics of this reaction are given later.

In a similar experiment with horse-blood catalase, the spectrum of complex II is also seen as shown by Fig. 5 (*B*). If the production of hydrogen peroxide is stopped by covering the solution with a layer of paraffin oil, the secondary complex slowly decomposes, and, after 1 hr., the spectrum of free catalase is obtained. The destruction of catalase during the entire experiment amounts only to less than 5 % as shown by curve *C* (Fig. 5).

A nearly identical Soret band is obtained when ascorbic acid is added to catalase. The data of Table 1 give the values of the extinction coefficient of the complexes formed from the notatin system or from ascorbic acid at pH $\approx$ 4. The data show the close similarity of the two Soret bands.

Spectroscopy of the primary and secondary catalase-hydrogen peroxide compounds in the visible region. As measured in the microspectroscope (Prof. Keilin made a number of these spectroscopic observations), the addition of 2 mM-glucose and 4×10^{-9} M-notatin to 10 μM-horse-liver catalase caused only a slight diminution of the catalase band at 629 mμ.; the combination of one of the catalase haematins with hydrogen peroxide to form the primary compound caused no other observable spectral shift (but see Fig. 6). After about 0·4 hr., the formation of the secondary catalase-hydrogen peroxide complex was complete, and the red colour and absorption bands of complex II at 536 and 572 mμ. were observed, the band at 629 mμ. having disappeared. Upon addition to catalase of ascorbic acid, which on autoxidation forms hydrogen peroxide, a nearly identical spectrum is observed; the absorption bands lie at 535 and 570 mμ. These bands are the same as those of the secondary compounds of catalase and alkyl hydrogen peroxides (Chance, 1949*c*), and thus this hydrogen peroxide complex has the same covalent iron-peroxide bonding and, as later data show, also lacks catalytic activity.

The proof that complex I has an absorption band beyond 653 mμ. As mentioned above, no shift of the absorption bands of catalase upon formation of complex I could be observed by visual spectroscopy. A definite shift of the catalase spectrum is indicated by Fig. 6. A sensitive spectrophotometer (Chance, 1947*b*) and a 1·3 cm. cuvette containing 8·2 μM-horse-liver catalase were used to record the relatively rapid formation of the primary catalase-hydrogen peroxide complex at 653 mμ., an isobestic point between the catalase and catalase-hydrogen peroxide-II spectra. Fig. 6 clearly shows the formation

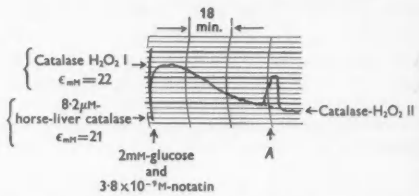


Fig. 6. The rapid formation of complex I of horse-liver catalase and its slow conversion into complex II registered at 653 mμ., an isobestic point between the spectra of catalase and complex II. The artifact at *A* is due to a temporary cut off of the electricity supply; pH 6·5, 0·01 M-phosphate.

of complex I on addition of notatin and glucose. A steady state ensues for some minutes, and then complex I disappears to form complex II. This record demonstrates for the first time that catalase-hydrogen peroxide has an absorption band in the red portion of the visible spectrum as has previously been observed with the primary peroxidase-hydrogen peroxide complex (Theorell, 1941).

The nature of the secondary catalase-hydrogen peroxide complex. In an experiment similar to that described above (Fig. 6), the formation of complex II is recorded in Fig. 7 at 435 mμ. by using appropriately diluted catalase. Upon addition of notatin to the mixture of glucose and catalase, there is an induction period during which the primary complex forms. No density change is, however, measured at 435 mμ. since catalase and complex I are isobestic at that wavelength. After this induction period, the reaction proceeds. The halftime for the formation of complex II is 780 sec., and its concentration reaches a maximum after about 0·5 hr.

To determine whether the reaction is reversible, 170 mM-ethanol was added and reduced the concentration of complex I instantly to very nearly zero because of the peroxidatic reaction between complex I and ethanol. This reduction in the concentration of complex I caused complex II to decompose slowly

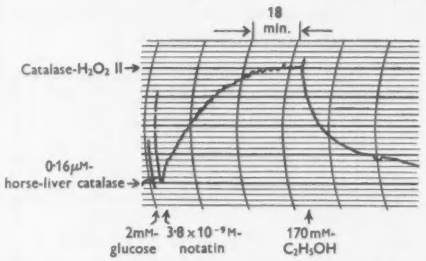


Fig. 7. The slow formation of complex II of horse-liver catalase at 435 mμ., an isobestic point between the spectra of catalase and complex I. The addition of ethanol causes the complex to disappear; pH 6·5, 0·01 M-phosphate.

with a halftime of 600 sec., corresponding to a first-order velocity constant of $1{\cdot}2 \times 10^{-3}$ sec.$^{-1}$ or a value of $k_4 = 7 \times 10^{-2}$ M^{-1} sec.$^{-1}$ which is of the same order of magnitude as obtained with the secondary catalase-alkyl hydrogen peroxide complexes (Chance, 1949*d*). The secondary catalase-hydrogen peroxide change in its concentration. Thus the secondary complex is stable in the presence of an excess of hydrogen peroxide and is, therefore, an inhibitor of catalase activity. The addition of ethanol to the complex formed in the presence of ascorbic acid causes the complex to decompose exactly as in Fig. 7.

The Soret band of complex II given in Fig. 4 (*B*), is not so high as that of catalase-methyl hydrogen peroxide II at 435 mμ. $\epsilon_{mx}^{435} = 210$ and the shape of the band and the position of its maximum indicates that not all catalase haematins were converted into the secondary complex under these conditions. This is demonstrated by reactions which were carried out in an open cuvette and which are summarized in Fig. 8. Record *A* shows that catalase cyanide forms very rapidly on adding cyanide to catalase. At 435 mμ., the optical density change is 26 scale divisions. In Record *B*, the secondary catalase-hydrogen peroxide complex is formed as in Fig. 7 with a smaller amplifier gain. Upon addition of cyanide to this complex, the density increment is 15·5 scale divisions which corresponds to the free catalase haematins. Thus $\frac{26-15{\cdot}5}{26} \times 3{\cdot}3 = 1{\cdot}3$ haematin groups are bound to peroxide. In contrast to the previous experiments where hydrogen peroxide bound to catalase in complex I prevents cyanide from attaching to one catalase haematin (Chance, 1949*b*), cyanide can replace peroxide from complex II here: the velocity

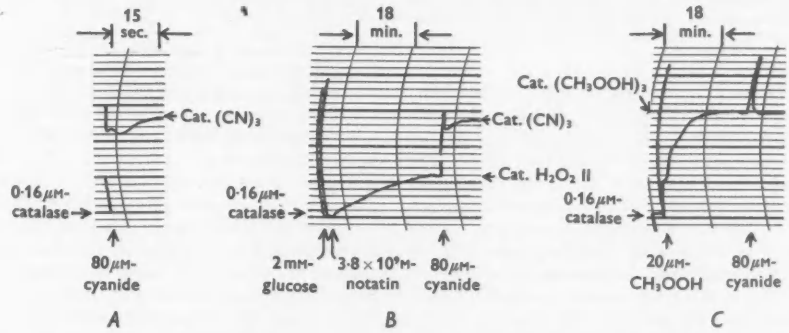


Fig. 8. A demonstration that not all catalase haematins are combined as complex II. Record *A*, the optical density change on addition of cyanide to free catalase. Record *B*, the formation of catalase-hydrogen peroxide II followed by the addition of cyanide. Record *C*, the formation of catalase-methyl hydrogen peroxide followed by the addition of cyanide. This horse-liver catalase was found to have 3·3 intact haematin groups. pH 6·5; 0·01 M-phosphate; λ = 435 mμ.

complex is therefore nearly completely inactive towards ethanol in the peroxidatic reaction. (The value of k_4 for the active primary complex is 10^3 M^{-1} sec.$^{-1}$ (Chance, 1947*a*).)

The addition of a large excess of hydrogen peroxide to the secondary complex has been found to cause no of combination of the rather large concentration of cyanide (80×10^{-6} M) with catalase

$$(80 \times 10^{-6} \times 9 \times 10^5 = 72 \text{ sec.}^{-1})$$

greatly exceeds the velocity of combination of the very dilute peroxide (approx. 10^{-9} M) (see p. 397) with

catalase (approx. $10^{-9} \times 3 \times 10^{7} = 0{\cdot}03$ sec.$^{-1}$). Thus these 1·3 haematins are bound as complex II. Record *C* (Fig. 8) shows a control experiment in which the secondary catalase-methyl hydrogen peroxide complex is formed, and, as the data indicate, the spectrophotometric change is larger and no haematin groups are free to combine with cyanide. Since the extinction coefficient of the catalase-alkyl hydrogen peroxide complex and the secondary catalase-hydrogen peroxide complexes are probably the same per haematin-group bound to peroxide (Chance, 1949*c*), $\frac{1}{2}\frac{2}{9} \times 3{\cdot}3 = 1{\cdot}4$ haematins are bound to peroxide in the secondary catalase-hydrogen peroxide complex. Thus only 1·4 catalase haematins have been converted into the secondary catalase-hydrogen peroxide complex at this value of pH.

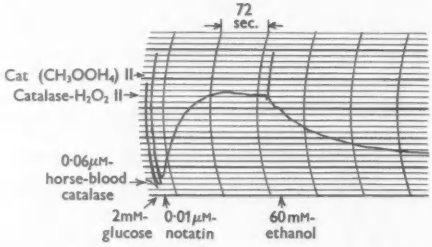


Fig. 9. The formation of the secondary catalase-hydrogen peroxide complex at pH 3·5. Note that time scale is fifteen times faster than in Fig. 7. The phosphate buffer was acidified with phosphoric acid to give pH 3·5 (final phosphate concentration 0·001 M); $\lambda = 435$ mμ.

However, Fig. 9 shows that at pH 3·5, the formation of the secondary catalase-hydrogen peroxide complex occurs much more rapidly than at pH 7·0 and involves nearly all of the catalase haematins. The halftime for formation of the complex is 29 sec. compared with 780 sec. at pH 7·0. Its decomposition time upon addition of ethanol is also smaller; 65 compared with 600 sec. In this case, the addition of ethanol does not liberate catalase to the extent obtained in Fig. 7. Also, the density change on formation of the secondary complex is very nearly as large as that shown for the formation of the secondary catalase-methyl hydrogen peroxide complex: $\frac{3}{3}\frac{2}{8} \times 4 = 3{\cdot}4$ of the 4 erythrocyte catalase haematins are bound to peroxide.

In acid solutions, the more rapid formation of the secondary complex and the greater extent of the reaction is in excellent agreement with the rapid and large decrease of catalase activity as measured by the rate of decomposition of hydrogen peroxide (Bonnichsen *et al.* 1947).

The inhibition of catalase activity by the secondary catalase-peroxide complexes. In order to demonstrate that the formation of the secondary complex inhibits the destruction of hydrogen peroxide by catalase experiments similar to that shown in Fig. 7 were carried out and samples were withdrawn, and, after suitable dilution (to about 1×10^{-9} M), were tested for catalase activity by the method described previously (Chance, 1949*a*). At pH 6·5, complex II is stable for a long interval (see Fig. 7) and is here assumed to be unaffected by the 100-fold dilution.

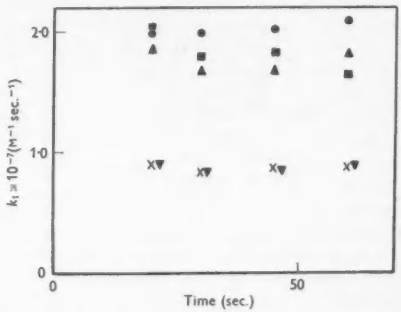


Fig. 10. The inhibition of catalase activity by the formation of the secondary complex at pH 6·5; 1·6 μM-horse-blood catalase mixed with 6×10^{-9} M-notatin and 2 mM-glucose. After the formation of complex II the solution is diluted to $5{\cdot}4 \times 10^{-10}$ M-catalase, 5 mM-hydrogen peroxide being added. Determination of k_1 gives the values represented by $\times$▼, k_1 being calculated from the equation $k_1 = (2{\cdot}3/et) \log x_0/x$, where e = M-concentration of catalase, t = time (sec.), x_0 and x = initial concentration of hydrogen peroxide and concentrations of hydrogen peroxide at subsequent times t. The untreated control solution gave points ■. Twelve min. after the addition of 0·25 mM-ethanol to the solution containing complex II, the activity test is repeated as shown by the points ▲. The corresponding effect upon the control is shown by the points ●.

The lower set of points, $\times$▼ (Fig. 10), shows that formation of complex II clearly inhibits the destruction of hydrogen peroxide by catalase. The spectrophotometric data show that about 1·7 haematins of the 4 horse-erythrocyte catalase haematins are converted into complex II at pH 6·5 (Fig. 8), and Fig. 10 shows that the relative decrease of activity is about 50 %. The reduction in activity over the range covered by these data is roughly proportional to the decrease in the number of haematins. Fig. 10 also shows that the inhibition caused by complex II can be largely reversed by the addition of ethanol in accordance with the spectrophotometric data of Fig. 7. In this case, several hours elapsed between the complete formation of complex II and the activity test. Thus the catalase solutions have lost considerable activity due to an irreversible reaction occurring on standing as indicated by the control experiments.

Table 2. *Relation between the inhibition of catalase activity and the amount of catalase-hydrogen peroxide II*

(Spectrophotometric test at $\lambda = 435$ mμ.: 0·17 μM-catalase, 4 mM-glucose, 5×10^{-9} M-notatin. Complete conversion to the complexes gives extinction increments corresponding to 42 and 40 scale divisions on the record respectively in the two experiments below. Activity test: $1{\cdot}7 \times 10^{-9}$ M-catalase, 6 mM-hydrogen peroxide, pH 6·2, $\lambda = 215$ mμ., Exp. 254. k_1 is the bimolecular velocity constant for peroxide decomposition in the equation: rate of decomposition $= k_1$ [catalase] $[H_2O_2]$. Cat. = catalase.)

Time after adding notatin (min.)	0	8	17	23	30	40
Horse-liver catalase						
Extinction increment at 435 mμ. (scale divisions) (Cat. $(CH_3OOH)_2$ II = 42 scale divisions)	0	11	13	23	27	29
Activity k_1 (M^{-1} sec.$^{-1} \times 10^{-7}$)	1·1	0·95	0·94	0·74	0·68	0·55
Horse-blood catalase						
Extinction increment at 435 mμ. (catalase $(CH_3OOH)_4$ II = 40 scale divisions)	0	—	—	—	—	28
Activity k_1 (M^{-1} sec.$^{-1} \times 10^{-7}$)	4·15	—	—	—	—	2·05

The progressive decrease of activity during the formation of complex II followed by withdrawing samples from the spectrophotometric test is shown in Table 2. This particular liver catalase had a rather low initial value of k_1.

In the case of horse-blood catalase, the activity is also reduced to about half the initial value owing to the binding of somewhat more than half the catalase haematins as complex II. It is very interesting that a decrease of the number of free haematins by about half causes a reduction of the activity by about half for both the horse-blood and horse-liver catalase containing four and less than four intact haematins respectively.

In acid solutions, the initial rapid decrease of catalase activity has already been demonstrated (Chance, 1948) and according to the data of Fig. 11, this loss of activity is correlated with the formation of the inactive secondary complex. In a spectrophotometric test at pH 4·0, the halftime for the formation of complex II is 3 min. Curve *A* (Fig. 11) shows that the decrease in the activity of catalase during the activity test by a solution of hydrogen peroxide occurs at about the same speed. The extent of conversion of catalase into complex II is in the spectrophotometric test $\frac{22}{46} \times 4 = 2$ haematins. The activity which was then measured in presence of notatin and glucose, as shown in curve *B* (Fig. 11), has a smaller initial value than the final value of curve *A*. Thus hydrogen peroxide from the notatin system produces a somewhat larger concentration of complex II than does a solution of hydrogen peroxide. Since the dissociation velocity of complex II is more rapid at this pH, the activity increases as shown in curve *B* and approaches the final values of curve *A*.

The addition of ascorbic acid to catalase has already been shown to produce a compound very similar to complex II, and curve *C* shows that the resulting inhibition of catalase activity is slightly greater than that obtained with the notatin system, in accord with a somewhat greater optical density change measured spectrophotometrically upon addition of ascorbic acid to catalase at this pH.

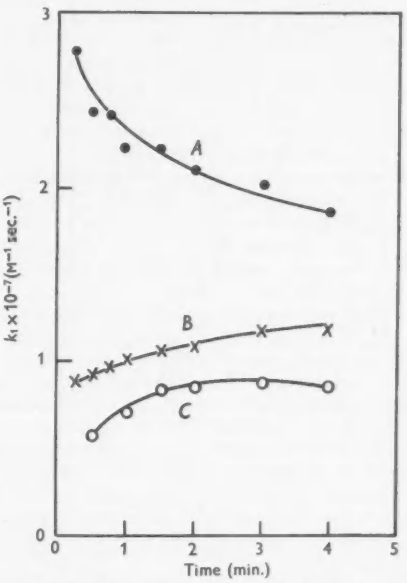


Fig. 11. The inhibition of catalase activity by the formation of complex II at pH 4·0. Curve *A*, activity of untreated horse-blood catalase (1×10^{-9} M) in the presence of 5 mM-hydrogen peroxide. Curve *B*, activity of horse-blood catalase after the formation of complex II in the presence of 4 mM-glucose and 5×10^{-9} M-notatin. Curve *C*, activity of horse-blood catalase after the formation of complex II in the presence of 1 mM-ascorbic acid. All curves in 0·001 M-phosphate buffer acidified with phosphoric acid to give pH 4·0.

At pH 3·6, the formation of complex II is nearly complete in a minute with either a solution of

Table 3. *Inhibition of catalase activity by the secondary complexes at* pH 3·6

(Spectrophotometric test at 435 mμ.: 0·056 μM-horse-blood catalase. Activity test: $1–2 \times 10^{-8}$ M-catalase, 5 mM-hydrogen peroxide; $\lambda = 215$ mμ.; k_1 is the bimolecular velocity constant for peroxide decomposition in the equation: Rate of decomposition $= k_1$ [catalase] $[H_2O_2]$. Activity of catalase at pH 6·2; $k_1 = 3{\cdot}5 \times 10^{-7}$ M^{-1} sec.$^{-1}$.)

	k_1 at 60 sec. after starting the activity test (M^{-1} sec.$^{-1} \times 10^{-7}$)	Extinction increment at 435 mμ. (scale divisions)	Halftime for formation of the complex (sec.)
Free catalase	1·24	—	—
Catalase + notatin, glucose, and oxygen (as in Table 2)	0·48	27	38
Catalase + methyl hydrogen peroxide (40 μM)	0·15	29	38
Catalase + ascorbic acid and oxygen (1 mM)	0·22	34	220

hydrogen peroxide in the activity test or with the notatin system or methyl hydrogen peroxide in the spectrophotometric test. The extinctions of complex II and the values of k_1, as determined in the activity test, are given in Table 3. The activity of untreated catalase is only one-third of the initial value after 60 sec. exposure to a solution of hydrogen peroxide in the activity test in accordance with previous data (Chance, 1948). The activity of the catalase solutions previously exposed to the notatin system or to methyl hydrogen peroxide is very low.

Since ascorbic acid is very slowly autoxidizable at this pH, the halftime for the formation of complex II is rather large because of the slow rate of production of hydrogen peroxide. Nevertheless, the conversion of catalase into complex II and the inhibition of catalase activity are very large.

In these experiments, as in those summarized in Fig. 11, the activity of catalase treated with the notatin system, methyl hydrogen peroxide or ascorbic acid increases during the activity test indicating a partial dissociation of complex II under the conditions of the activity test.

At pH 3·6, it is possible to form spectroscopically measurable amounts of complex II by direct addition of a solution of (bottle) hydrogen peroxide to catalase, several additions of dilute hydrogen peroxide give about 20 % of the amount formed in Table 3 by using the notatin system.

The reaction kinetics of catalase-hydrogen peroxide I in the presence of the notatin system. Not only has the notatin system led to the discovery of the secondary complex just described, but this system has been very useful in the study of the primary complex of catalase and hydrogen peroxide, not only for the determination of its spectra, but also in studies of the mechanism of catalase action.

In the rapid-flow apparatus (Fig. 12), the formation of the primary complex when a solution of hydrogen peroxide is added (left) is compared with that from hydrogen peroxide formed by the notatin system (right). The record with the solution of hydrogen peroxide begins with the observation tube filled with free catalase. On initiating the flow of reactants, the primary complex is formed completely at the shortest time measured due to the rapidity of the combination of catalase and 200 μM-hydrogen

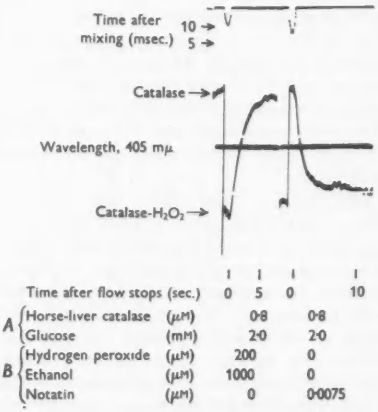
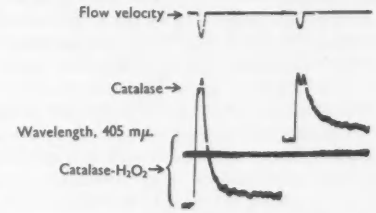


Fig. 12. A comparison of the formation of catalase-hydrogen peroxide I in the presence of a solution of hydrogen peroxide and ethanol (left) and hydrogen peroxide produced from notatin, glucose and oxygen (right). Ethanol causes the rapid disappearance of the primary complex in the left-hand record. Solutions *A* were mixed with solutions *B* in the rapid-flow apparatus; pH 6·5, 0·01 M-phosphate.

peroxide. The complex remains at a constant concentration until the flow has stopped. Then the reaction of the primary complex and ethanol gives free catalase after several seconds. With the notatin system, the record begins with the observation tube filled with the primary complex. On initiating the flow, the observation tube is filled with free catalase, and no formation of the primary complex is seen until the flow has stopped. Then the primary complex

forms in several seconds and remains saturated with hydrogen peroxide produced by the notatin system. The great difference in the speed of formation of the primary complex in these two records is caused by the great difference in the hydrogen peroxide concentration. Not only does the notatin system furnish a small amount of hydrogen peroxide, but the catalase also combines with the peroxide and decomposes it. Nevertheless, the formation of the primary complex goes very nearly to completion because the hydrogen peroxide is continuously generated.

The steady-state concentration of complex I is very sensitive to the presence of ethanol as shown by Fig. 13, where the addition of only 20 μM-ethanol reduces the steady-state concentration of complex I to about one-half the value obtained without



Time after flow stops (sec.)			0	10	0	10
A	Horse-liver catalase	(μM)	0·8		0·8	
	Glucose	(mM)	2·0		2·0	
B	Notatin	(μM)	0·0075		0·0075	
	Ethanol	(μM)	0		20	

Fig. 13. The reduction in the steady-state concentration of catalase-hydrogen peroxide I upon the addition of 20 μM-ethanol (right-hand record). Solutions *A* were mixed with solutions *B* in the rapid-flow apparatus; pH 6·5, 0·01 M-phosphate.

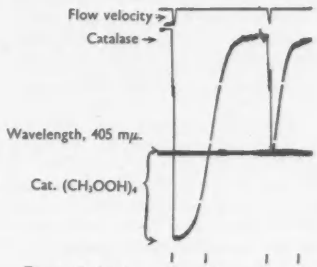
ethanol. For these conditions, the rate of reaction of complex I with ethanol is roughly equal to the rate of formation of complex I. Numerically, the rates of these reactions are equal to the products, pk_4a and $\frac{1}{2}ek_1x$ respectively, where k_4 is the velocity constant for the reaction of the complex I (p) with ethanol (a), and k_1 is the velocity constant for the reaction of catalase (e) with hydrogen peroxide (x) (see Chance, 1948, equations 3 and 4). If these are the only two reactions involved, their rates are equal in the steady state and $x = k_4a/k_1$, since $\frac{1}{2}e = p$. Since $k_1 = 3 \times 10^7$ M^{-1} sec.$^{-1}$ and $k_4 = 1 \times 10^3$ M^{-1} sec.$^{-1}$ (Chance, 1947*a*), the value of x is about

$$\frac{10^3 \times 20 \times 10^{-6}}{3 \times 10^7} = 7 \times 10^{-10} \text{M}.$$

This is, however, a minimum value since the 'catalatic' reaction, the reaction of complex I with a second molecule of peroxide (Chance, 1948), is neglected. A mathematical analysis on the basis of two possible mechanisms for these reactions shows that the error caused by this rough approximation is not very large. Complete details will be given later.

The reaction of hydrogen peroxide with catalase-methyl hydrogen peroxide I. In another paper (Chance, 1949*e*), it was shown that methyl hydrogen peroxide groups bound to catalase haematin react with a solution of hydrogen peroxide. It is now possible to study this interesting reaction in more detail by using hydrogen peroxide generated continuously by the notatin system.

In Fig. 14, the formation and decomposition of the catalase-methyl hydrogen peroxide complex requires more than 10 sec. in the presence of ethanol; but if



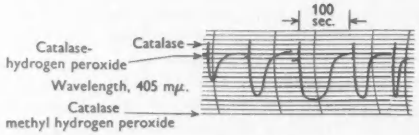
Time scale (sec.)		0	10	0 10
Horse-liver catalase	(μM)	1·6		1·6
Methylhydrogen peroxide	(μM)	26		26
Ethanol	(μM)	400		400
Notatin	(μM)	0		0·42
Glucose	(μM)	2·0		2·0
$-dO_2/dt$	(μM/sec.)	0		5·0
$k_3 = x_0/(P_{max}\ t_j)$	(sec.$^{-1}$)	0·4		8·2

Fig. 14. The reaction of catalase-methyl hydrogen peroxide I with hydrogen peroxide continuously generated by the notatin system. The left-hand record shows the kinetics of catalase-methyl hydrogen peroxide I in the presence of ethanol only; in the right-hand record the notatin system is present. Catalase, glucose and ethanol are mixed with methyl hydrogen peroxide and notatin. The oxygen consumption was measured by the platinum micro-electrode; pH 6·5, 0·01 M-phosphate.

hydrogen peroxide is generated at the rate of 5 μM/sec. by the notatin system, there is a dramatic decrease in the saturation value and in the halftime for decomposition of the complex. The value for k_3 calculated by the formula used previously (Chance, 1949*d*), has increased to 8·2 sec.$^{-1}$, equivalent to the addition of about 10 mM-ethanol. Thus this record demonstrates the action of hydrogen peroxide as an acceptor according to the mechanism discussed previously (Chance, 1949*e*).

In Fig. 15, the methyl hydrogen peroxide and notatin concentrations are varied in order to study the reaction more closely. Since ethanol is omitted, the observation tube is initially filled with the catalase-hydrogen peroxide complex as in Fig. 12.

On initiating the flow of reactants, the extinction of free catalase is obtained, and as the flow stops, the methyl hydrogen peroxide combines with the catalase haematins and then reacts with the hydrogen peroxide giving values of k_3 which clearly increase with the notatin concentration and with the rate of oxygen uptake ($-dO_2/dt$), and decrease slightly with



Notatin ($M \times 10^6$)	0·76	0·76	0·76	1·9	7·6
Methyl hydrogen peroxide (μM)	1·2	2·4	4·8	4·8	4·8
dO_2/dt (μM/sec.)	0·028	0·028	0·028	0·07	0·20
$k_3 = X_0/(P_{max}\ t_{\frac{1}{2}})$ (sec.$^{-1}$)	0·076	0·048	0·045	0·13	0·39
$\frac{k_3}{dO_2/dt} \times 10^6$ (M^{-1})	2·7	1·7	1·6	1·9	1·4

Fig. 15. The effect of notatin and methyl hydrogen peroxide concentrations upon the reaction of catalase-methyl hydrogen peroxide I and hydrogen peroxide; 0·8 μM-horse-liver catalase and 2·0 mM-glucose are mixed with varying amounts of notatin and methyl hydrogen peroxide. The blank rate is $k_3 = 0{\cdot}008$ sec.$^{-1}$. The oxygen uptake was measured by the platinum micro-electrode; pH 6·5, 0·01 M-phosphate.

the methyl hydrogen peroxide concentration. The proportionality between the values of k_3 and the rate of oxygen uptake is indicated by the constancy of their quotient given on the bottom row of the Table in Fig. 15.

By using the value of free hydrogen peroxide concentration estimated from the data of Fig. 13 (approx. 10^{-9} M) for $0{\cdot}76 \times 10^{-9}$ M-notatin, the value of the velocity constant for the reaction of catalase-methyl hydrogen peroxide I and hydrogen peroxide is $\frac{k'_3}{[H_2O_2]} = \frac{0{\cdot}048}{1 \times 10^{-9}} = 5 \times 10^7\ M^{-1}$ sec.$^{-1}$. As already stated, the value of the hydrogen peroxide concentration is not very accurate. Nevertheless, the magnitude of the velocity constant strongly suggests that hydrogen peroxide reacts with catalase-bound methyl hydrogen peroxide as rapidly as with catalase-bound hydrogen peroxide.

DISCUSSION

The use of the notatin system for studying catalase has verified and extended the earlier studies of the properties of the primary catalase-hydrogen peroxide complex in which a solution of (bottle) hydrogen peroxide was used. The Soret band of complex I has been accurately measured and verifies the earlier data obtained in the 1 mm. circular capillary observation tube of the rapid-flow apparatus which employs a wider spectral interval (approx. 7·5 mμ., Chance, 1947*a*). The values of the change of millimolecular extinction coefficient ($\Delta\epsilon_{mM}^{405}$) at 405 m$\mu$. on formation of complex I are 45 and 48 cm. for a horse-liver catalase (about 3·3 haematins) and for a horse-blood catalase (4 haematins) respectively. These values agree to within the accuracy with which the catalase concentrations are known. These values are about 10 % larger than those obtained previously, as would be expected from the smaller spectral interval which can be used in these experiments (approx. 0·5 mμ.).

In a previous paper, the change of ϵ_{mM}^{405} per catalase haematin bound to methyl hydrogen peroxide was given as 45 (Chance, 1949*c*). Reasoning as in that paper, the composition of catalase-hydrogen peroxide I is, to within the experimental error, one catalase haematin bound to peroxide. The experimental error is at least 1 % of the total extinction measured ($\epsilon_{mM}^{405} = 380$, error = 4) which gives the value of $1{\cdot}0 \pm 0{\cdot}1$ peroxide-bound haematins. This apparently conflicts with the value, $1{\cdot}2 \pm 0{\cdot}1$ peroxide-bound haematins, previously obtained (Chance, 1949*b*). The accuracy of the two methods is about the same, and the cause of the discrepancy is not known.

Since the applicability of a particular catalase mechanism depends upon whether this value differs from exactly one, the accuracy of these determinations is still inadequate for a final decision.

The existence of an absorption band of catalase-hydrogen peroxide I beyond 653 mμ. has finally been demonstrated spectrophotometrically. Attempts to observe this band visually were, however, unsuccessful since the change of extinction coefficient is very small. This type of absorption band which was first observed in the primary peroxidase-hydrogen peroxide complex (Theorell, 1941) is clearly seen in the primary catalase-alkyl hydrogen peroxide complexes (Chance, 1949*c*) where all of the intact catalase haematins are bound and is now established as being characteristic of the primary complexes.

The sensitivity of the available apparatus in the region above 660 mμ. was not sufficient to locate the peak of the band of catalase-hydrogen peroxide I with accuracy. The absorption band of catalase-alkyl hydrogen peroxide I lies at 670 mμ. Since the isobestic points between catalase-hydrogen peroxide I and catalase, and catalase-alkyl hydrogen peroxide II and catalase lie at about the same wavelengths, 653 and 650 mμ. respectively, the peak of the band of catalase-hydrogen peroxide I probably also lies at 670 mμ. (Chance, 1949*c*).

When a solution of hydrogen peroxide is added to catalase, no formation of the secondary catalase-hydrogen peroxide complex has been observed spectroscopically in any previous study. The formation of this secondary complex, like that of the alkyl hydrogen peroxides, is slow; the probability of the conversion from the primary to the secondary com-

plex is small, and the formation of significant amounts of the latter requires that the steady-state concentration of the primary complex be maintained for some time. As previous records clearly show, a steady concentration of catalase-hydrogen peroxide I occurs for only a few tenths of a second when a solution of hydrogen peroxide is used, and this explains why complex II has not previously been detected spectroscopically. However, with any system which produces hydrogen peroxide continuously at a rate sufficient to saturate catalase with hydrogen peroxide, the formation of complex II is readily observed spectroscopically. Thus complex II, which was first identified using the notatin system, has also been formed in the presence of ascorbic acid and could probably be demonstrated using any of the other hydrogen peroxide-producing oxidase systems studied by Keilin & Hartree (1936, 1945).

The kinetics of formation of the secondary complex have not been studied in detail. However, the rate increases with an increase in the rate of hydrogen peroxide production and with a decrease in the pH. The quantitative evaluation of a reaction velocity constant is difficult because the concentration of free hydrogen peroxide can at present only be measured from the kinetics of complex I in the presence of ethanol as described above. The rate of formation of the secondary catalase-alkyl hydrogen peroxide complexes was found to decrease with decreasing peroxide concentration. Therefore, the comparatively slow formation of the secondary catalase-hydrogen peroxide complex shown in Fig. 7 is in accord with the very small value of the free hydrogen peroxide concentration estimated from the results of Fig. 13 (approx. 10^{-9} M). One may venture to calculate a velocity constant on the basis of the halftime for formation of complex II given in Fig. 7 and this hydrogen peroxide concentration

$$\frac{0{\cdot}7}{730 \times 1 \times 10^{-9}} = \sim 10^{6}\,\text{M}^{-1}\,\text{sec.}^{-1}.$$

The approximate value thus obtained is larger than the values found with the alkyl hydrogen peroxides.

The number of catalase haematins which are bound as complex II varies with the experimental conditions. Using the notatin system, about half the catalase haematins are bound at pH 6·5 and nearly all at pH 3·6 under the same experimental conditions. On the other hand, with ascorbic acid nearly all the catalase haematins are bound at pH 6·5 and 3·6. Furthermore, the conditions for the tests of catalase activity give, in acid solutions, less of the complex II than is formed from the notatin system or ascorbic acid. There is, therefore, an equilibrium between the primary and secondary complexes which depends upon the pH and the manner in which the hydrogen peroxide is produced.

That the secondary complex is in equilibrium with the primary complex has already been suggested by studies of the catalase-alkyl hydrogen peroxides, and is supported by these studies where the removal of the primary complex by the addition of ethanol causes a slow dissociation of the secondary complex. Keilin & Hartree (1945) give excellent confirmation of the fact that complex II will not form when complex I can react peroxidatically with ethanol; in their cyclic oxidations (see their Fig. 2), the activity of catalase continues with no measurable decrease for several hours.

The mechanism by which catalase haematins are progressively converted from complex I to complex II is not yet known. It is suggested that, as soon as a particular haematin-peroxide bond changes from complex I to complex II, one of the remaining free catalase haematins combines with peroxide to form complex I and so on until equilibrium conditions are reached.

There are several reasons for concluding that Lemberg & Foulkes's (1948) catalase-ascorbic acid compound is actually catalase-hydrogen peroxide complex II. The complex I has already been shown to be formed from hydrogen peroxide produced from ascorbic acid oxidation. These studies show that complex I is converted into complex II in the presence of a continuous supply of hydrogen peroxide which can be obtained by oxidation of ascorbic acid. The visible absorption bands and the Soret bands of the complexes formed from ascorbic acid or from the notatin system show great similarity. Both complexes decompose in the presence of ethanol. In both cases, the activity of catalase is inhibited and is here shown to be inhibited in proportion to the amount of complex formed as determined spectrophotometrically at 435 mμ.

There are, however, two discrepancies: (1) the position of the β bands differ by 2 mμ. (Lemberg & Foulkes (1948) find the β band to lie at 565 mμ., a discrepancy of 7 mμ.); and (2), the amount of complex II formed in the presence of ascorbic acid considerably exceeds that formed in the presence of the notatin system at pH 7, but not at pH 3·5–4·0. It is possible that the equilibrium between the primary and secondary complexes is affected by the ascorbic acid molecule itself and thereby both the β-band position and the amount of complex II would be affected.

The discovery of complex II is a most fortunate event for the study of the destruction of hydrogen peroxide by catalase. For the first time, a direct explanation is provided for the inactivation of catalase in the determination of Kat. f. The exponential decrease of reaction velocity constant during the course of the activity determinations by the method of von Euler & Josephson (1927) is in accord with the measured first-order kinetics of

formation of complex II. It has been possible to make measurements of the concentration of complex II and of the activity of this catalase solution containing complex II. These studies clearly demonstrate that complex II is an inhibitor of catalase activity, and permit the explanation of a large number of the phenomena associated with catalase activity which have not previously been fully understood.

At neutral pH, the halftime for the formation of complex II is shown in Fig. 7 to be about 10 min. When the activity of complex I is measured over the first minute only, then a negligible amount of complex II forms, and constant activity is obtained and affords a better method for the measurement of catalase activity (Bonnichsen *et al.* 1947). The success of this method depends only upon measuring the activity before complex II forms; the increase of catalase concentration to about 10^{-9}M is needed only to increase the accuracy of measuring the reaction-velocity constant titrimetrically. This method fails, however, at pH 3·5 where complex II has nearly completely formed in the first minute of the reaction.

In the use of this method, the velocity of the destruction of hydrogen peroxide by catalase increases linearly with the peroxide concentration, at least up to 0·3M-hydrogen peroxide, and the 'Michaelis constant' found by earlier workers can now be attributed to the increase in the formation of complex II at the higher peroxide concentrations. This effect not only counterbalances the increase of reaction velocity with peroxide concentrations and gives a plateau, but it also causes a decrease in reaction velocity in very high hydrogen peroxide concentrations. It is very likely that complex II is not the only factor causing these effects, probably some irreversible enzyme inactivation occurs.

In their analysis of the effect of hydrogen peroxide concentration upon catalase activity, Lineweaver & Burke (1934) have postulated an inactive enzyme-substrate complex of catalase and hydrogen peroxide; but their mechanism was based upon the supposed Michaelis constant for catalase action of 0·025M and, therefore, does not represent a formulation of these reactions.

George (1947, 1948, 1949) has studied 'rapid' and 'slow' phases of catalase activity in the presence of very high hydrogen peroxide concentrations. As measured by a rapid manometric technique, the activity of catalase is found to decrease rapidly for about 2 min. at pH 5·85. Then the activity of catalase is relatively constant. The experiments of this paper suggest that the initial decrease of catalase activity is caused by the formation of complex II and that the constant activity thereafter is caused by the catalase haematins not bound as complex II. In fact, a 'peroxide complex having a lower catalytic activity' was postulated by George to explain his results. A number of the effects found by George can be explained qualitatively on the basis of the lack of activity of complex II, and the changes in equilibrium between complex I and complex II caused by hydrogen peroxide concentration and by inhibitors. However, this explanation is not yet applied quantitatively to his experiments for the following reasons: (1) the initial activity of catalase measured by George is only 1·0–2·5% of that found in these tests; (2) the rate of decrease of activity in George's tests is more rapid than the rate of formation of complex II measured spectrophotometrically in the presence of the notatin system; (3) a quantitative study of the equilibrium of complex I and complex II has not yet been made.

Bonnichsen (1948) has recently studied the relation between the number of intact haematin groups in bile pigment-containing catalases and their activity. The formation of complex II affords a much simpler method of decreasing the number of active catalase haematins of a given catalase.

Since the number of catalase haematins bound to peroxide as complex II increases from about half the total number at pH 6·5 to nearly all at pH 3·6, these studies can be made over a wider range than was possible in Bonnichsen's (1948) work. The decrease of activity during the formation of complex II at pH 5–6·5 can also be studied by withdrawing samples from the solution in which complex II is forming and testing their activity. These exploratory experiments clearly show the decrease of activity caused by the formation of complex II and show rough proportionality between activity and number of free haematins. At pH 6·5, about half the catalase haematins are bound as complex II, and a 50% decrease of the activity is obtained for horse-blood and horse-liver catalase preparations; a reduction from 4 to 2 active catalase haematins causes about the same decrease in activity as a reduction from 3 to 1 active haematin. This result does not agree with that found by Bonnichsen (1948), where it was concluded that more than 1 free catalase haematin was required for catalase activity. There are, however, large differences in the experimental method; Bonnichsen reduced the number of active haematins by bile pigment formation. Here the bile pigment content is not increased; the haematins are only bound in an inactive peroxide complex.

Highly accurate studies of this relationship between the number of active haematin groups and the catalase activity require an apparatus in which catalase activity and the concentration of complexes I and II can be measured simultaneously and in the same solution. Such experiments require a method about 100 times more sensitive or more rapid than that used in the present studies.

Continuously generated hydrogen peroxide has been very useful in studies of the mechanism of catalase action. With a solution of hydrogen peroxide, it is possible to obtain a steady state only in the concentration of the primary catalase-hydrogen peroxide complex for a few tenths of a second; the hydrogen peroxide concentration itself is decreasing rapidly. With continuously generated hydrogen peroxide, a steady state in both the concentration of complex I and the hydrogen peroxide concentration is obtained for several minutes. Under these conditions, the complicated differential equations for the various catalase mechanisms can be readily solved. This 'double' steady state has been achieved by continuous flow devices (Stead, Page & Denbigh, 1948), but it has not been previously recognized that it could be accomplished enzymically. The solutions of these equations, which will be presented in a later paper, indicate that the approximations used here to calculate the free hydrogen peroxide concentration cannot be far from correct values. By observing that the saturation of the primary catalase-hydrogen peroxide complex, formed in the presence of the notatin system, could be halved by the initiation of the peroxidatic reaction on the addition of a very small amount of ethanol under the particular experimental conditions of this coupled oxidation, it has been possible to estimate the free hydrogen peroxide concentration to be about 10^{-9} M. This low hydrogen peroxide concentration is characteristic of coupled oxidations.

Calculations based on this value give the velocity constant for the reaction of hydrogen peroxide with catalase-methyl hydrogen peroxide I of the same order of magnitude as that required for the reaction with catalase-hydrogen peroxide I in the usual 'catalatic' reaction. The similarity of these two reaction velocity constants is in accord with the suggestion that hydrogen peroxide is here acting as an acceptor; other data show that the velocity of reaction of the acceptor molecule with the enzyme-substrate compound is usually unaffected by whether hydrogen peroxide or alkyl hydrogen peroxides are bound to catalase haematin. The theory of catalase action which postulates a peroxidatic reaction between the primary catalase-hydrogen peroxide complex and hydrogen peroxide, receives support from these experiments. It is interesting that such a small hydrogen peroxide concentration causes such a rapid reaction; in Fig. 13 approx. 10^{-9} M-hydrogen peroxide caused the same reaction velocity as would have 5×10^{-5} M-ethanol.

In this reaction, hydrogen peroxide is acting as a reducing agent, whereas it acts as an oxidizing agent in the oxidation of alcohols by catalase hydrogen peroxide. The range of possible modes of action of catalase peroxides in biological systems are: (1) the breakdown of hydrogen peroxide into water and oxygen in which hydrogen peroxide molecules are both oxidant and reductant; (2) the oxidation of substances similar to the lower alcohols, methylene glycol and formic acid with (*a*) hydrogen peroxide as substrate, (*b*) alkyl hydrogen peroxides as substrates; (3) the oxidation of alkyl hydrogen peroxides with hydrogen peroxide as reductant.

SUMMARY

1. With hydrogen peroxide continuously generated by the notatin system, an absorption band beyond 653 mμ. in the spectrum of the primary catalase-hydrogen peroxide complex (complex I) has been found and this completes the spectroscopic analogy between catalase-peroxide and peroxidase-peroxide complexes in which this type of absorption band has previously been observed by visual spectroscopy to lie at 670 mμ.

2. In the presence of the notatin system, the Soret band of the primary catalase-hydrogen peroxide complex has been measured in the ordinary spectrophotometer and agrees substantially with that obtained previously by using the flow method and a solution of (bottle) hydrogen peroxide.

3. The change of millimolecular extinction coefficient from catalase to catalase-hydrogen peroxide I at 405 mμ. *i.e.* $\Delta\epsilon_{\text{mM}}^{405}$ is 48 for horse-erythrocyte catalase which agrees to within the experimental error with the value for a horse-liver catalase. On comparison with the value for catalase-methyl hydrogen peroxide I, the composition of catalase hydrogen peroxide I is $1{\cdot}0 \pm 0{\cdot}1$ catalase haematins bound to hydrogen peroxide in the primary complex.

4. With the notatin system, the conversion of the primary catalase-hydrogen peroxide complex into an inactive form (complex II) has been observed. This complex has visible absorption bands at 536 and 572 mμ., and is analogous to the inactive secondary catalase-alkyl hydrogen peroxide complexes except that the number of catalase haematins bound as complex II varies with the experimental conditions.

5. Complex II is formed in the presence of hydrogen peroxide produced by the autoxidation of ascorbic acid or by the notatin system. The catalase-ascorbic acid compound of Lemberg & Foulkes (1948) is probably complex II. A small amount of complex II has been observed spectroscopically by direct addition of a solution of hydrogen peroxide to dilute catalase.

6. The rate of formation of complex II increases with an increase in the steady-state hydrogen peroxide concentration and with a decrease in pH.

7. As determined by the rate of destruction of hydrogen peroxide, the activity of catalase partially converted into complex II is proportional to the number of free haematins. At pH 6·5, about half the

catalase haematins are bound as complex II and at pH 3·6 nearly all are, under the particular experimental conditions.

8. The formation of complex II is responsible for the decrease of catalase activity during the usual Kat. f. determination and for the rapid decrease of catalase activity in acid solutions.

9. The steady-state concentration of hydrogen peroxide in the presence of catalase and the notatin system is very small. Under particular experimental conditions, the hydrogen peroxide concentration is calculated to be of the order of 10^{-9} M.

10. The very rapid reaction of catalase-bound methyl hydrogen peroxide I with hydrogen peroxide can be studied in greater detail when hydrogen peroxide is continuously generated by the notatin system. The velocity constant for this reaction is of the same order as that of catalase-hydrogen peroxide I with hydrogen peroxide ($3{\cdot}5 \times 10^7$ M^{-1} sec.$^{-1}$). In this reaction, hydrogen peroxide acts as an acceptor as in peroxidatic reactions of complex I with ethanol, but is about 30,000 times as active as ethanol.

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The Enzyme-substrate Compounds of Bacterial Catalase and Peroxides*

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One of the approaches to the relation between enzyme activity and the nature of the protein component is the study of enzymes with dissimilar protein components and identical prosthetic groups. A comparative study of the peroxidatic activity of horse radish peroxidase and ferrimyoglobin affords a gross example of the potent influence of the protein component. But here the effect is too great; ferrimyoglobin is practically completely inactive. A more sensitive investigation of the differences between the activities of erythrocyte catalase and the recently crystallized bacterial catalase (Herbert & Pinsent, 1948) would seem to be more fruitful, and is the subject of this paper.

Both erythrocyte and bacterial catalases apparently have four protohaematin prosthetic groups attached to protein molecules of very nearly the same molecular weight. However, Herbert & Pinsent (1948) obtained definite evidence that differences exist between the two protein components, especially as indicated by their relative stability in organic solvents and at low values of pH. There is also good evidence that the activity of bacterial catalase is different from that of erythro-

* Paper No. 14 of a series on Catalases and Peroxidases.

cyte catalase (the values of 'Katalasefähigkeit' (Kat. f.) found by the above authors were 95,000 and 61,000 respectively). Thus these two catalases are suitable for a quantitative study of the effect of the protein component upon the activity of the two enzymes.

This paper gives detailed data on the spectroscopic and kinetic properties of the compounds of bacterial catalase with hydrogen peroxide and with alkyl hydrogen peroxides. The data show that the mechanism of action of this enzyme is identical with that of erythrocyte catalase. However, the reaction velocity constants for the combination of bacterial catalase with peroxides and for the reactions of the bacterial catalase-peroxide complexes with alcohols and related substances show significant differences from those of erythrocyte catalase; bacterial catalase reacts more rapidly with small molecules and more slowly with larger molecules than erythrocyte catalase. The difference between the activities of these two enzymes may thus be attributed to a difference of protein structure. It is suggested that the haematins of bacterial catalase are more deeply 'buried' in the protein molecule than are those of erythrocyte catalase, and reactions with larger molecules are thus impeded. But the activity of the haematins of bacterial catalase towards the small hydrogen peroxide molecule is enhanced by the proximity of the protein component and the prosthetic groups.

EXPERIMENTAL

Light absorption measurements. Unless otherwise stated, a Beckman quartz spectrophotometer was used, and all results are given in terms of the millimolecular extinction coefficient $\epsilon_{mM} = E/cl$ (l.mmol.$^{-1}$ cm.$^{-1}$), where E is the extinction ($\log I_0/I$), c the mM-concentration and l the optical depth in cm. Wavelengths (in mμ.) are indicated by superscripts, e.g. ϵ_{mM}^{405}. In the case of catalase, c is expressed in the units mol. catalase/l. and *not* mol. catalase-haematin/l. as employed by some workers.

Preparations. Bacterial catalase was prepared from *Micrococcus lysodeikticus* by the method previously described (Herbert & Pinsent, 1948). To conserve material, the preparations were not carried through to crystallization, and represented the material obtained after stage 7 of the above procedure; they were only about 60–70% pure, but were free from other haematin compounds.

Enzyme concentrations. The content of pure catalase in solutions of the approx. 60% pure enzyme was determined by (*a*) haematin determinations by the pyridine haemochromogen method, (*b*) spectrophotometric measurement of the Soret absorption band of catalase at 405 mμ.

(*a*) Pyridine haemochromogen was determined by spectrophotometric measurements at two wavelengths, the peak of the α absorption band and the minimum between the α and β bands. According to recent measurements by Biörck (1949) with the Beckman spectrophotometer, these lie at 556 and 540 mμ. respectively, and the difference between the molecular extinction coefficients is

$$(\epsilon^{556} - \epsilon^{540}) = 22{\cdot}0 \times 10^3.$$

Hence, when measurements are made in an optical depth of 1 cm.,

$$\text{Haematin Fe concentration (mM)} = (E^{556} - E^{540})/22{\cdot}0.$$

These values are slightly different from those obtained by Drabkin (1941), using a different type of instrument; since our measurements were made with a Beckman spectrophotometer, Biörck's data were used.

(*b*) The millimolecular extinction coefficient of bacterial catalase at the peak of the Soret band was obtained by measuring the absorption at 405 mμ. of solutions standardized by method (*a*), and is $\epsilon_{mM}^{405} = 405 \pm 5$. This value was thereafter used for routine determinations of catalase concentration. It is 6–7% higher than the corresponding values for erythrocyte catalase given by Bonnichsen (1949), and Bonnichsen, Chance & Theorell (1947). For these bacterial catalase preparations, the value of ϵ^{280} is 1·5 to 2 times that of ϵ^{405}.

RESULTS

Activity towards hydrogen peroxide

In previous work on bacterial catalase the activity was expressed as Kat. f., and measured by the titration method of Sumner (1941), using catalase concentrations of approximately 0·006 μg./ml. which gave a halftime for the reaction of about 10 min. By this method, the value found for Kat. f. was 95,000 (Herbert & Pinsent, 1948). Bonnichsen *et al.* (1947) pointed out the disadvantages of this technique, the main one being that considerable destruction of enzyme by the hydrogen peroxide occurs, giving falling values of the velocity constant k_1', which must be extrapolated to zero time to obtain the true value, an unsatisfactory procedure. The activity of bacterial catalase was therefore redetermined by the rapid titration method of the above authors, using considerably higher enzyme concentrations (approx. 0·12 μg./ml.) which gave a halftime of approx. 30 sec. Under these conditions, no appreciable destruction of enzyme occurred and the value of k_1' remained satisfactorily constant throughout the reaction.

Under such conditions, the destruction of hydrogen peroxide by catalase follows exactly the equation (Bonnichsen *et al.* 1947)

$$-\frac{dx}{dt} = k_1' e x, \qquad (1a)$$

and k_1' is calculated from the titration results as

$$k_1' = \frac{1}{et} \ln \frac{x_o}{x_t}, \qquad (1b)$$

where e and x are the molar concentrations of catalase and hydrogen peroxide, and t is the time in sec. Kat. f. is related to k_1' by the equation:

$$\text{Kat. f.} = \frac{520\, k_1'}{M}, \qquad (1c)$$

where M is the molecular weight of bacterial catalase (230,000).

Table 1. *Determinations of k'_1 by spectrophotometric measurements at* 230 mμ.

($1{\cdot}06 \times 10^{-9}$ M-bacterial catalase, pH 7·0, 0·01 M-phosphate. 330 μM-Hydrogen peroxide added.)

Initial recorder deflexion	...	...	21	29	29	30	26	23
Halftime of the reaction (sec.)	...	...	12·6	12·4	12·4	10·3	14·8	11·8
$k'_1 = \dfrac{\ln 2}{(\text{Average } t_{0{\cdot}5}) \times 1{\cdot}06 \times 10^{-9}}$ (M^{-1} sec.$^{-1}$)			$5{\cdot}3 \times 10^7$					

A rather careful series of measurements by the rapid titration method gave, at 0°,

$$k'_1 = 4{\cdot}30 \times 10^7 \text{ M}^{-1} \text{ sec.}^{-1},$$

or Kat. f. = 97,300, a slight increase on the previous value.*

Since measurements in the rapid-flow apparatus cannot conveniently be made at 0°, the change in k'_1 with temperature was measured by the rapid titration method over the range 0–30°; the results are given in Fig. 1. The unusually low temperature coefficient taxes the accuracy of the titration method, but within the experimental error the results appear to follow the Arrhenius equation, giving a linear plot of log k'_1 against $1/T$, and a value for the apparent activation energy (μ) of 1410 cal. This is appreciably lower than the value obtained for erythrocyte catalase by Bonnichsen *et al.* (1947), who found $\mu = 1700 \pm 100$ cal.

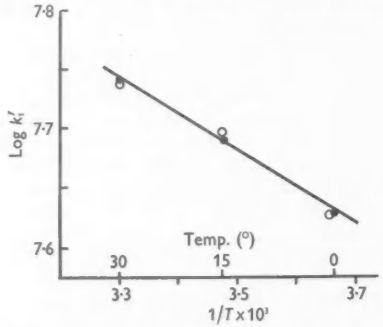

Fig. 1. Temperature coefficient of bacterial catalase in the range 0–30°. Determinations by the rapid titration method. Final concentrations: hydrogen peroxide, 0·005 M; catalase, $4{\cdot}76 \times 10^{-10}$ M; phosphate (pH 6·8), 0·01 M. Reaction halftime approx. 30 sec. Two experiments with different enzyme solutions, each at three temperatures. Each point is the mean of quadruplicate determinations. Temp. in °C., T in °K.

The activity of bacterial catalase has also been determined by a new method in which the disappearance of hydrogen peroxide is followed spectrophotometrically by measurement of its absorption at 230 mμ. By appropriate modification of the electrical circuits of the Beckman spectrophotometer adequate stability may be obtained in the measurement of small changes of optical density $\log I_0/I = 0{\cdot}01$ approx. (Chance, unpublished data). Thus it is possible to record directly, and on a linear scale, the decrease of hydrogen peroxide concentration during the catalase reaction. Furthermore, a special form of the 'stopped-flow' method has recently been developed which is suitable for use in the ultraviolet region of the spectrum. The observation chamber of this apparatus has a depth of 1 cm.

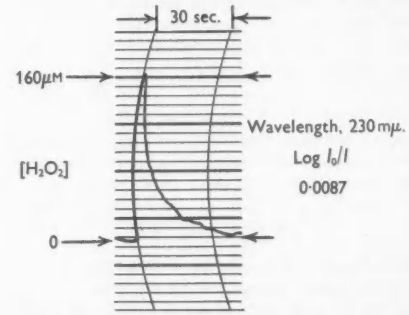

Fig. 2. Illustrating the determination of the velocity constant for the disappearance of hydrogen peroxide in the presence of bacterial catalase. The measurement of hydrogen peroxide concentration is made spectrophotometrically at 230 mμ. Modified Beckman spectrophotometer, stopped-flow method ($d_0 = 1$ cm.). Final concentrations: $2{\cdot}8 \times 10^{-9}$ M-bacterial catalase, 0·01 M-phosphate, pH 7·0. Temp. 25°.

A typical example of such a measurement at 230 mμ. is given in Fig. 2. Initially the observation chamber is filled with the end products of a previous identical experiment in which all the hydrogen peroxide has been consumed. On initiating the flow of reactants, the observation chamber is filled with mixed bacterial catalase ($2{\cdot}8 \times 10^{-9}$ M)† and hydrogen peroxide (160 μM)† and the increment in the concentration of the latter is indicated by the rapid upward deflexion of the trace. The disappearance of hydrogen peroxide proceeds and causes the exponential fall of

* $4{\cdot}30 \times 10^7$ M^{-1} sec.$^{-1}$ corresponds to $4{\cdot}30 \times 10^7$ l.mol.$^{-1}$ sec.$^{-1}$ in the alternative terminology.

† Here and throughout this paper, concentrations referred to are the final concentrations in the reaction mixture.

the trace. The halftime of the reaction is measured (4·7 sec.) and k_1' is computed:

$$k_1' = \frac{\ln 2}{4{\cdot}7 \times 2{\cdot}8 \times 10^{-9}} = 5{\cdot}3 \times 10^7 \text{M}^{-1} \text{ sec.}^{-1} \text{ (at 25°)}.$$

Similar experiments with $1{\cdot}06 \times 10^{-9}$M-enzyme give the same value for k_1' (Table 1). In comparison, the value of k_1' at 25° determined by the rapid titration method is $5{\cdot}35 \times 10^7 \text{M}^{-1} \text{ sec.}^{-1}$ (equivalent to a Kat. f. of 121,000).

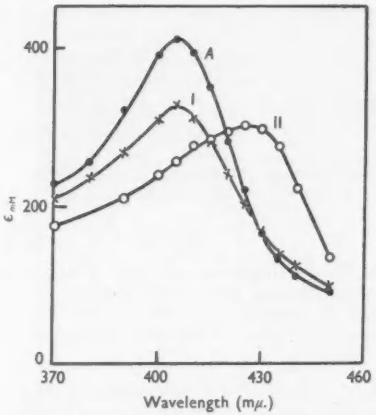

Fig. 3. The spectra of the primary and secondary catalase-hydrogen peroxide complexes in the region of the Soret band. Curve *A*, free bacterial catalase, 0·5 μM, in phosphate (pH 7·0), 0·01 M. Curve I, bacterial catalase-hydrogen peroxide complex I. Catalase concentration as above, hydrogen peroxide supplied by the notatin system (approx. 10^{-9}M-notatin, 0·7 mM-glucose). Curve II, bacterial catalase-hydrogen peroxide complex II, measured 60 min. after mixing bacterial catalase and the notatin system.

The excellent agreement between these values, obtained by two different observers using quite different methods, speaks for the fundamental soundness of both methods, and verifies that the activity of bacterial catalase is considerably greater than that of erythrocyte catalase, where the values of k_1' are $3{\cdot}5 \times 10^7$ at 25° (determined by both methods) and $2{\cdot}63 \times 10^7$ at 0°.

Spectroscopic studies of the bacterial catalase-peroxide complexes

An intermediate compound of enzyme and substrate was discovered previously in the reaction of erythrocyte catalase and hydrogen peroxide by the use of the rapid-flow method (Chance, 1947), and these studies show that a very similar compound exists in the reaction of bacterial catalase and hydrogen peroxide. Such a complex may be stabilized for a considerable time if hydrogen peroxide is continuously generated by an oxidase system which produces hydrogen peroxide, for example, the system notatin, glucose and oxygen (Keilin & Hartree, 1948). In the presence of this enzyme system (and in the absence of ethanol) the spectrum of the primary bacterial catalase-hydrogen peroxide complex (complex I) is measured in the Beckman spectrophotometer in the region 370–455 mμ., and the values of the extinction coefficients are plotted in Fig. 3.

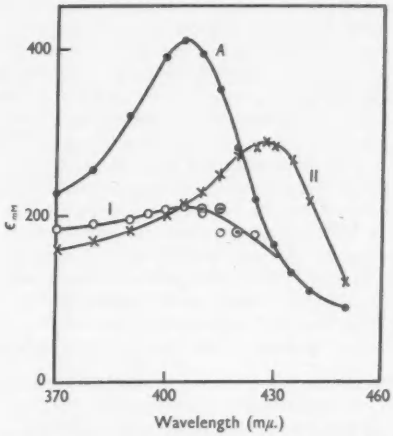

Fig. 4. The spectra of the primary and secondary bacterial catalase-methyl hydrogen peroxide complexes in the region of the Soret band. Curve *A*, free bacterial catalase. Curve I, bacterial catalase-methyl hydrogen peroxide complex I. Curve II, bacterial catalase-methyl hydrogen peroxide complex II. Final concentrations, 0·5 μM-bacterial catalase in 0·01 M-phosphate, pH 7·0. Curve I, rapid-flow apparatus; circles, 600 μM-methyl hydrogen peroxide; points, 5 μM-methyl hydrogen peroxide. (Exp. 414.) Curve II, Beckman spectrophotometer, 200 μM-methyl hydrogen peroxide.

The Soret band of complex I of bacterial catalase is in many respects very similar to that found for erythrocyte catalase (Chance, 1949*g*); it is similar to, but less intense than, that of the free enzyme. But there is a quantitative difference: the change of $\epsilon_{\text{mM}}^{405}$ from the free enzyme to complex I is considerably larger, 82 compared with 45 for erythrocyte catalase (Chance, 1950*a*). This difference is discussed further below.

After 1 hr. in the presence of hydrogen peroxide, the spectrum II of Fig. 3 is obtained. This represents the conversion of most of the bacterial catalase into the secondary complex, in accordance with studies of erythrocyte catalase (Chance, 1950*a*).

Bacterial catalase also forms primary and secondary compounds with methyl hydrogen peroxide as

shown by the curves I and II of Fig. 4. The spectrum of the secondary complex is readily measured in the ordinary spectrophotometer upon the addition of an excess of methyl hydrogen peroxide (approx. 200 μM) to bacterial catalase (approx. 0·5 μM). The primary complex is, however, unstable, and either spontaneously decomposes into free catalase or is converted into the secondary complex in a short time. The spectrum of complex I is therefore measured in the rapid-flow apparatus. The changes of extinction coefficient from free catalase to complex I and from complex I to complex II are measured according to the method described previously (Chance, 1949*c*). In the region 370–420 mμ., the extinction coefficients based upon the conversion of complex I to complex II are considered the more accurate, while those based upon the conversion of catalase to complex I are considered more accurate in the region above 420 mμ. In this way the spectrum of Fig. 4 is constructed. It was noted that the values of the change of extinction coefficient from free catalase to complex I in the region 380–410 mμ. lie somewhat above the curve based upon the change from complex I to complex II given in Fig. 4. This discrepancy was observed previously (Chance, 1949*c*), and is again attributed to the large spectral interval required of the monochromator employed with the rapid-flow apparatus.

The composition of the bacterial catalase-hydrogen peroxide complex: spectrophotometric data

The change of ϵ_{mM}^{405} from free catalase to the primary methyl hydrogen peroxide complex (200) is much larger than that shown in Fig. 3 for the hydrogen peroxide complex (82). Since this change is identical for the primary peroxidase hydrogen peroxide and methyl hydrogen peroxide complexes (Chance, 1949*g*), it is here assumed that the change of extinction coefficient from the free enzyme to the primary peroxide complex is the same per haematin group for hydrogen peroxide and methyl hydrogen peroxide. Thus the smaller value for the bacterial catalase-hydrogen peroxide complex may be attributed to the fact that not all the catalase haematins are bound to hydrogen peroxide. Quantitatively, only $82/200 = 1{\cdot}6$ of the four catalase haematins are so bound.

A similar effect obtains in erythrocyte catalases, but here $1{\cdot}0 \pm 0{\cdot}1$ haematins are bound according to studies by a similar method (Chance, 1950*a*). Thus there is a higher steady-state concentration of the catalase-hydrogen peroxide complex in the reaction of bacterial catalase than in the reaction of erythrocyte catalase with hydrogen peroxide.

A graphic illustration of the difference of the extinction coefficients of the hydrogen peroxide and methyl hydrogen peroxide complexes is given in Fig. 5. The cuvette ($d = 1$ cm.) of a recording spectrophotometer is initially filled with a dilute solution of bacterial catalase. As a control the solution is initially stirred, and, except for the momentary increase of optical density caused by stirring, the base-line is unaffected. Upon addition of hydrogen peroxide to a concentration of 67 μM, an abrupt decrease of extinction is recorded and is caused by the formation of the primary catalase-hydrogen peroxide complex. The addition of hydrogen peroxide is repeated and causes no further decrease of extinction. This indicates that the saturation value of the catalase-hydrogen peroxide complex

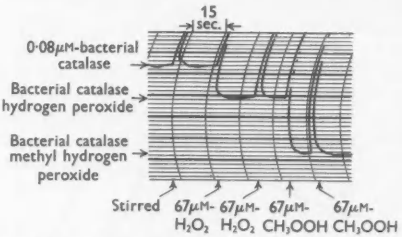

Fig. 5. Spectrophotometric recording of the reactions of bacterial catalase with hydrogen peroxide and with methyl hydrogen peroxide. Modified Beckman spectrophotometer, 1 cm. cuvette; 0·01 M-phosphate, pH 7·0; $\lambda = 405$ mμ.

has been measured. The next addition is of methyl hydrogen peroxide which combines with all the catalase haematins and thereby causes a large decrease of extinction. Further addition of methyl hydrogen peroxide causes no increase in the concentration of catalase-methyl hydrogen peroxide. Since saturation with peroxide has been achieved in these two cases, and since it is assumed that the extinction coefficients of the two peroxide compounds are the same on a haematin-iron basis, the fraction of catalase haematins bound in the hydrogen peroxide complex is 7·5/20·5 scale divisions of Fig. 5. For a four-haematin catalase this corresponds to 1·5 haematins bound. This is in fair agreement with the value of 1·6 measured in Fig. 3.

Cyanide results

The composition of the mammalian catalase-hydrogen peroxide complexes has been determined by measuring spectrophotometrically the relative amounts of catalase-cyanide formed by the free catalase or by the catalase-hydrogen peroxide complex (Chance, 1949*b*). In those tests, conditions were specified under which a successful experiment could be performed. These conditions involved the dissociation constant of catalase-cyanide. For this reason, the properties of bacterial catalase-cyanide are given. The spectrum of bacterial catalase-cyanide in the region 370–460 mμ. is shown in Fig. 6, and is seen to be very similar to that of erythrocyte

catalase-cyanide (Chance, 1949*c*). ϵ_{mM}^{425} is 360 and is 13% greater than the corresponding value for erythrocyte catalase-cyanide.

The titration of bacterial catalase with cyanide is illustrated in Fig. 7, and the values of the apparent dissociation constant are computed by the usual formula. The average is $21{\cdot}8 \times 10^{-6}$ M. This is over five times the value found for the equilibrium constant of erythrocyte catalase and cyanide (Chance, 1949*a*). This change is apparently not caused by a difference in the rates of formation of the cyanide compounds; measurements of this value for bacterial catalase give about 2×10^6 M^{-1} sec.$^{-1}$ compared with $0{\cdot}9 \times 10^6$ for mammalian catalases (Chance, 1949*a*).

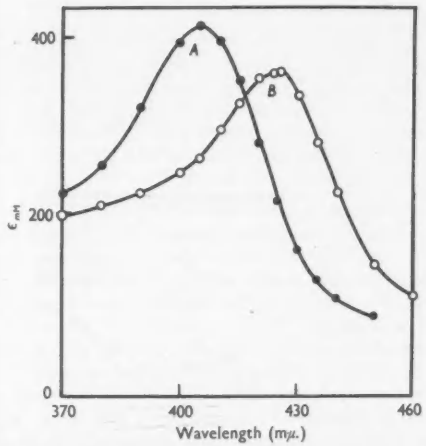

Fig. 6. The spectrum of bacterial catalase-cyanide in the region of the Soret band (Curve *B*). Final concentrations: 0·4 μM-bacterial catalase, 400 μM-hydrocyanic acid, 0·01 M-phosphate, pH 7·0. Curve *A*, free bacterial catalase for comparison.

Thus it is the velocity constant for the dissociation of bacterial catalase-cyanide that is increased greatly: from the product of the dissociation constant and the velocity constant for the formation of catalase-cyanide a value of 44 sec.$^{-1}$ is computed for the velocity constant for the dissociation of bacterial catalase-cyanide. This is about eleven times that for erythrocyte catalase-cyanide and is comparable to the dissociation velocity of oxyhaemoglobin.

With higher concentrations of hydrocyanic acid (approx. 20 μM), the combination of cyanide with the free haematin groups of the bacterial catalase-hydrogen peroxide complex is clearly shown in Fig. 8*A* on a slow-time scale and in Fig. 8*B* on a fast-time scale. These measurements were made in the rapid-flow apparatus. On initiation of the flow, the catalase-cyanide remaining from the previous experiment is flushed out of the observation tube and is replaced by fresh catalase, hydrogen peroxide, and hydrocyanic acid as indicated by the abrupt decrease in optical density. The catalase-hydrogen peroxide complex is not measured at this wavelength because it forms very rapidly, and because 425 mμ. is near the isobestic point for catalase and catalase-hydrogen peroxide (Fig. 3). Thus the extinction in the observation tube is nearly the same as that of free catalase. The rapid upward deflexion (D_2) as the flow stops is caused by the combination of catalase and cyanide, and this reaction, according to Fig. 8*B*, is complete in about 0·1 sec. But Fig. 8*A* clearly shows that the upward deflexion continues at a slower rate until a constant value E_1 is reached. The slow reaction is caused by the decomposition of catalase-hydrogen peroxide which liberates catalase haematin

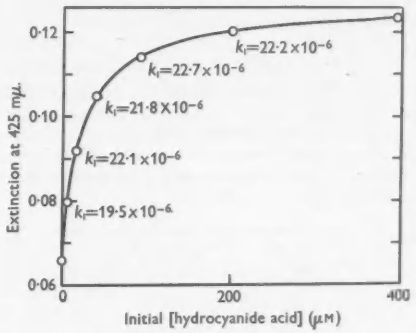

Fig. 7. Titration of bacterial catalase with hydrocyanic acid. The values of the dissociation constant K_1 are computed for the various points. Final concentrations: 0·4 μM-bacterial catalase, 0·01 M-phosphate, pH 7·0; λ=435 mμ.

to combine with cyanide. Thus E_2/E_1 is a measure of the catalase haematins not bound to hydrogen peroxide in this complex, and $1-E_2/E_1$ a measure of the catalase haematins bound to hydrogen peroxide. Fig. 8 is, therefore, clearly in agreement with the previous tests which show that not all the catalase haematins are bound to hydrogen peroxide in this complex.

Fig. 9 represents the effect of cyanide concentration upon the value of $1-E_2/E_1$ and clearly shows a decrease in this quantity with increasing cyanide concentration. This demonstrates that cyanide may compete with peroxide for catalase haematin. However, this competition interferes with the accurate measurement of the maximum value of $1-E_2/E_1$, which would be obtained at very low cyanide concentrations. Such data are difficult to obtain with bacterial catalase because of the large dissociation constant of the cyanide complex (22×10^{-6} M) which necessitates a relatively large excess of hydro-

cyanic acid to give readily measurable amounts of catalase cyanide. Therefore the maximum value of $1-E_2/E_1$ is obtained by extrapolation of the data of Fig. 9 to zero hydrocyanic acid concentration, as indicated by the dotted portion of the curve. A value of about 0·39 is obtained, which corresponds to about 1·6 haematins bound to hydrogen peroxide for a four-haematin catalase.

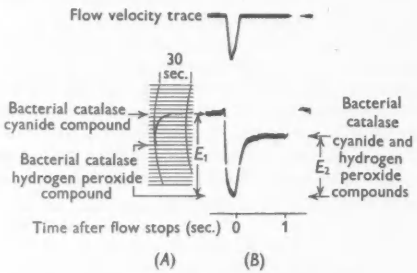

Fig. 8. The simultaneous reactions of hydrogen peroxide and hydrocyanic acid with bacterial catalase on a slow-time scale (*A*) and on a fast-time scale (*B*). Final concentrations: 0·045 μM-bacterial catalase, 200 μM-hydrogen peroxide, 20 μM-hydrocyanic acid, 4 mM-methanol, 0·01 M-phosphate, pH 7·0; $\lambda = 435$ mμ.

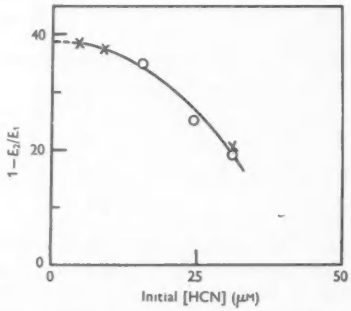

Fig. 9. The relation between the quantity $1-E_2/E_1$ (see Fig. 8) and the initial hydrocyanic acid concentration. The decrease of $1-E_2/E_1$ with increasing hydrocyanic acid concentration illustrates competition between peroxide and cyanide for catalase haematin. Conditions as in Fig. 7.

The fact that it is necessary to obtain the value of $1-E_2/E_1$ by extrapolation in the case of bacterial catalase shows that the definition of the experimental conditions for a successful experiment for erythrocyte catalase by this method (Chance, 1949*c*) is inadequate for bacterial catalase. We can point to one inadequacy of the previous analysis: the criterion of whether cyanide would displace hydrogen peroxide from complex I was based upon the initial hydrogen peroxide concentration. However, the initial hydrogen peroxide concentration will have been greatly reduced by the catalatic reaction by the time the cyanide begins to combine with the catalase, and competition may indeed occur with more dilute cyanide than would otherwise be expected. This situation is aggravated by the fact that bacterial catalase has a higher activity towards hydrogen peroxide, and a larger dissociation constant of the cyanide complex, than has erythrocyte catalase. It is therefore recommended that the relation between $1-E_2/E_1$ and cyanide concentration be investigated for each new catalase for which this test is used.

The relation between the kinetics of disappearance of hydrogen peroxide and the kinetics of the catalase-hydrogen peroxide complex

Numerous attempts have previously been made to devise methods for the measurement of the kinetics of the catalase-hydrogen peroxide complex at the same time as the kinetics of the disappearance of hydrogen peroxide (Chance, 1947). By the development of suitable electronic circuits and a stopped-flow method suitable for measurements in the ultraviolet region, the rapid disappearance of hydrogen peroxide can be measured with a fairly rapid response. This stopped-flow apparatus has an observation chamber with an optical path of 1·0 cm. and permits the measurement of the kinetics of the catalase-hydrogen peroxide complex in very dilute solutions (0·07 μM).

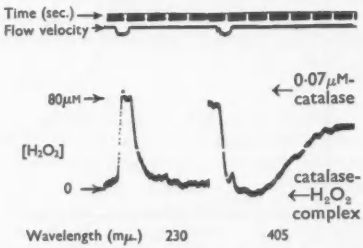

Fig. 10. Illustrating the relationship between the kinetics of disappearance of hydrogen peroxide measured at 230 mμ. and the kinetics of the bacterial catalase-hydrogen peroxide complex measured at 405 mμ. Modified Beckman spectrophotometer, stopped-flow method, cell depth 1 cm. Time markers are at 1 sec. intervals. Final concentrations: 0·07 μM-bacterial catalase, 80 μM-hydrogen peroxide, 8 mM-methanol, 0·01 M-phosphate, pH 7·0. (Exp. 416*b*.)

A preliminary record of this type is shown in Fig. 10 and represents the performance obtainable with the present technique operating near the limit of its sensitivity. At 230 mμ. is registered the kinetics of the disappearance of hydrogen peroxide, and the description given for Fig. 2 applies here except that the time scale is more rapid. At 405 mμ., the abrupt

downward deflexion is caused by the rapid combination of catalase and hydrogen peroxide. The complex remains at its saturation value for over 2 sec. and decomposes on reaction with methanol.

This record clearly shows that the hydrogen peroxide concentration must fall to a very small value (too small to be measured accurately) before the reaction of the catalase-hydrogen peroxide complex with methanol begins. Thus the previous statements based on less direct evidence are verified (Chance, 1947).

It is of considerable interest to compare this record with that of the reaction kinetics of a conventional Michaelis intermediate complex (Chance, 1943, Fig. 3; Chance, 1949*f*, Fig. 1), where the concentration of the peroxidase-hydrogen peroxide complex determines the rate of disappearance of hydrogen peroxide. With catalases, the rate of disappearance of hydrogen peroxide and the concentration of the enzyme-substrate compound are related in a much less obvious manner.

The combination of bacterial catalase with peroxides

Fig. 11 illustrates the measurement of the velocity of formation of the bacterial catalase-hydrogen peroxide complex by the rapid-flow method. The capillary observation tube is initially filled with free catalase, the end product of the previous identical experiment. On initiation of the flow of fresh reactants into the capillary (0·36 μM-catalase mixed with 10 μM-hydrogen peroxide and 8 mM-methanol), the rapid combination of catalase and hydrogen peroxide causes the rapid decrease in optical density. The reaction proceeds to over half completion in the time indicated by the top trace: the flow velocity attained corresponds to a time after mixing of about 3 msec. As the flow stops, longer times are measured and the reaction goes rapidly to completion. The catalase-hydrogen peroxide complex is not stable and its decomposition is indicated by the upward slope of the trace. After about 0·8 sec., the flow of reactants is re-initiated and there is an upward deflexion because catalase and hydrogen peroxide have not time to react completely at the time measured (approx. 2 msec.). As the flow stops, the reaction goes to completion. The catalase-hydrogen peroxide complex is then allowed to decompose completely, and the final extinction is indicated by the final mark on the record. This record illustrates the most satisfactory manner of measuring on one record the initial and final values of the reaction, as well as a few values of the extent of the reaction at various times after mixing. It is pointed out that initial and final values are especially difficult to measure in the catalase-hydrogen peroxide reactions, in which the total change in extinction is only a small portion of the extinction of the enzyme solution.

The velocity constant k_1 computed from this record is about 5×10^7 M^{-1} sec.$^{-1}$. The results of a series of similar measurements of the velocity of

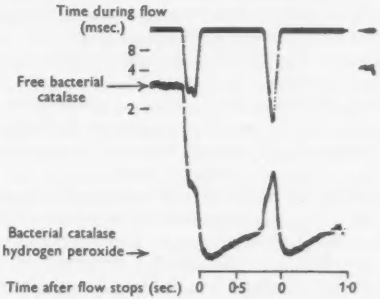

Fig. 11. Illustrating the measurement of the combination of bacterial catalase and hydrogen peroxide. Rapid-flow apparatus, $\lambda = 405$ mμ. Final concentrations: 0·34 μM-bacterial catalase, 10 μM-hydrogen peroxide, 8 mM-methanol, 0·01 M-phosphate, pH 7·0. Temp. 20°.

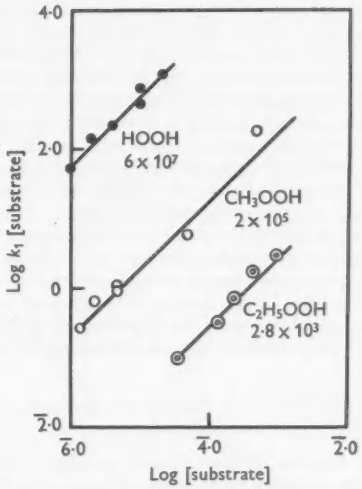

Fig. 12. The second-order combination of bacterial catalase and hydrogen peroxide, methyl hydrogen peroxide, and ethyl hydrogen peroxide, demonstrated by the 45° straight lines through the experimental points. Substrate concentrations are given in terms of molarity and the units of the reaction velocity constants are (shown as 6×10^7 etc.) M^{-1} sec.$^{-1}$. Rapid-flow apparatus, $\lambda = 405$ mμ. Final concentrations: 0·34 μM-bacterial catalase, 0·01 M-phosphate, pH 7·0. Temp. 20° for H_2O_2, 25° for $CH_3.OOH$ and $C_2H_5.OOH$.

combination of bacterial catalase and hydrogen peroxide are given in Fig. 12 where the graph of k_1 [substrate] versus [substrate] is plotted in log-

arithmic co-ordinates. A 45° straight line indicates a constant value of k_1 and a second-order reaction for the combination of bacterial catalase and hydrogen peroxide. The average value of the reaction-velocity constant k_1 is 6×10^7 M^{-1} sec.$^{-1}$. It is not possible, with present techniques, to demonstrate the validity of this second-order reaction over a wider range of hydrogen peroxide concentrations because of the extreme rapidity of the reaction; these data only cover the range 1–20 μM-hydrogen peroxide. Above 20 μM-hydrogen peroxide, the reaction proceeds too rapidly for accurate measurement; it is over 90% complete at the shortest time measured. Nevertheless, the values of k_1 measured under these conditions are less than 6×10^7 M^{-1} sec.$^{-1}$ and may indicate a deviation from the second-order reaction.

The data obtained in this experiment are more consistent than are those obtained from studies of mammalian catalase where the accuracy was inadequate to prove that the reaction is of the second order (Chance, 1947). These data are more accurate because the reaction of bacterial catalase and hydrogen peroxide gives a larger change of extinction than that of an equal concentration of mammalian catalase.

The values of the reaction velocity constants (k_1) for bacterial and mammalian catalases may be compared at similar hydrogen peroxide concentrations (4–10 μM), and are 6×10^7 and 3×10^7 M^{-1} sec.$^{-1}$ respectively. The velocity of combination of bacterial catalase with hydrogen peroxide is thus roughly twice that of mammalian catalase. This is in accord with the larger activity of bacterial catalase.

Fig. 12 indicates that bacterial catalase combines with methyl or ethyl hydrogen peroxide in true second-order reactions of velocity constants 2×10^5 and $2{\cdot}8 \times 10^3$ M^{-1} sec.$^{-1}$ respectively. These reactions are considerably slower than the corresponding reactions of mammalian catalase (Chance, 1949*d*).

The titration of bacterial catalase with hydrogen peroxide

The amount of the bacterial catalase-hydrogen peroxide complex decreases with decreasing hydrogen peroxide concentration, as shown by the relation between the initial hydrogen peroxide concentration and the oscillograph deflexion at 405 mμ. corresponding to the change in extinction from free catalase to the complex. The latter is measured as in Fig. 11, and the results are plotted in Fig. 13. A 'dissociation constant' may be computed on the basis that the saturation value of the complex corresponds to 1·6 out of 4 haematins or $0{\cdot}45 \times 1{\cdot}6 = 0{\cdot}72$ μM, and computations based on the data at higher peroxide concentrations give a value of about $0{\cdot}4 \times 10^{-6}$. As pointed out previously (Chance, 1947), the true dissociation constant is considerably smaller because a large portion of the initial hydrogen peroxide is decomposed during the formation of the complex.

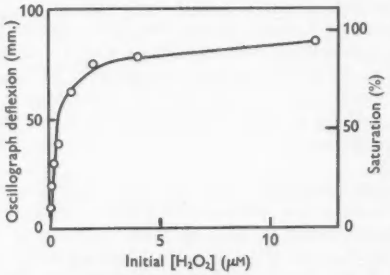

Fig. 13. The titration of bacterial catalase with hydrogen peroxide. Rapid-flow apparatus, $\lambda = 405$ mμ. Final concentrations: 0·45 μM-bacterial catalase, 4 mM-methanol, 0·01 M-phosphate, pH 7·0.

The spontaneous decomposition of the primary bacterial catalase-hydrogen peroxide complex

The velocity of this reaction is considerably less than that measured for mammalian catalases (Chance, 1947), and halftimes of roughly 200 sec. are measured, corresponding to a velocity constant of 0·0035 sec.$^{-1}$. Assuming that only the rate of formation and spontaneous decomposition are involved, the true dissociation constant of the bacterial catalase-hydrogen peroxide complex I may be as small as 6×10^{-10}.

The reaction of bacterial catalase peroxides with acceptors

The data of Fig. 14 give the results of measurements of the rate at which the catalase-hydrogen peroxide complex decomposes in the presence of various acceptors at various concentrations. Again the data are plotted on a logarithmic scale in order to cover the extreme range. The 45° slope of the straight lines indicates that the reaction of catalase hydrogen peroxide with the acceptors is of the second order and the position of the lines gives the values of the reaction velocity constants. The values of k_4 are, in general, smaller than the corresponding values for mammalian catalase (Chance, 1947; 1950*b*). However, the striking effect is the great decrease in reaction velocity with the size of the acceptor molecule; the velocity constant for ethanol is much less than that for methanol, and strong propanol gives a scarcely measurable reaction ($k_4 = 0{\cdot}025$ M^{-1} sec.$^{-1}$ approx.).

The primary bacterial catalase-methyl hydrogen peroxide complex also reacts with acceptors and, in this case, the values of k_4 are computed from measurements of the 'cycle' of the enzyme-substrate complex (Chance, 1949*d*). The measured values of k_4

for formate, methanol and ethanol are 150, 54 and 11 M^{-1} sec.$^{-1}$ respectively. The agreement with the data of Fig. 13 is fair except in the case of methanol where the value is smaller for the alkyl hydrogen peroxide complex.

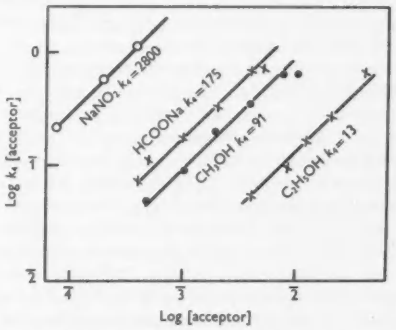

Fig. 14. The second-order reaction of bacterial catalase-hydrogen peroxide complex I with various acceptors. The 45° straight lines through the experimental points demonstrate the second-order reaction mechanism. Acceptor concentrations are given in terms of molarity and the units of the velocity constants are M^{-1} sec.$^{-1}$. Final concentrations: 0·185 μM-bacterial catalase, 80 μM-hydrogen peroxide, 0·01 M-phosphate, pH 7·0; λ=405 mμ.

The reaction of the primary catalase-alkyl hydrogen peroxide complex with formaldehyde (probably as methylene glycol) proceeds satisfactorily and k_4=270 M^{-1} sec.$^{-1}$. The difficulties in the measurement of the reaction of erythrocyte catalase-hydrogen peroxide with formaldehyde and erythrocyte catalase-methyl hydrogen peroxide with nitrite are recited elsewhere (Chance, 1950*b*) and are verified in these experiments.

DISCUSSION

The great similarity of erythrocyte and bacterial catalase indicated by the work of Herbert & Pinsent (1948) is apparently restricted to those properties of the enzymes which are principally determined by the chemical nature of their prosthetic groups and by the size of their protein components: haematin content, light absorption, sedimentation constant, etc. In addition, their enzymic functions and specificities are found to be generally the same.

Generally speaking, these studies extend the similarity of the two enzymes to the properties of their enzyme-substrate compounds. Bacterial catalase forms with hydrogen peroxide and with methyl or ethyl hydrogen peroxide primary and secondary compounds which have absorption spectra that are similar to those of the primary erythrocyte catalase-peroxide compounds. In addition, these compounds form rapidly and oxidize alcohols and related compounds. It is of special interest to note that the oxidation of acceptors by bacterial catalase and peroxides apparently involves only the green primary complex, in accord with previous studies of mammalian catalases. Superficially, the enzymes might be said to be practically identical. But Herbert & Pinsent (1948) found the value of activity (Kat. f.) towards hydrogen peroxide is larger for bacterial catalase than for erythrocyte catalase. This difference has been studied in some detail and is verified by these data which show that the velocity constant (k_1') for the disappearance of hydrogen peroxide in the presence of bacterial catalase is $5{\cdot}3 \times 10^7$ M^{-1} sec.$^{-1}$ compared with $3{\cdot}5 \times 10^7$ for erythrocyte catalase. This difference is related to two other differences revealed by these experiments: (1) The number of hydrogen peroxide molecules per bacterial catalase molecule in the primary complex is $1{\cdot}6 \pm 0{\cdot}1$, compared with $1{\cdot}0 \pm 0{\cdot}1$ found for erythrocyte catalase by similar methods. (2) The reaction-velocity constant (k_1) for the combination of bacterial catalase and hydrogen peroxide is about 6×10^7 M^{-1} sec.$^{-1}$ compared with about 3×10^7 for erythrocyte catalase under similar conditions. Thus the greater activity of bacterial catalase may be attributed to the more rapid combination with hydrogen peroxide which in turn causes a higher steady-state concentration of the catalase-hydrogen peroxide complex. Since the rate of disappearance of hydrogen peroxide is proportional to the steady-state concentration of the intermediate complex, the activity towards hydrogen peroxide, as measured in terms of Kat. f. or k_1', would be expected to be larger than the value for erythrocyte catalase which has a smaller steady-state concentration of the intermediate complex.

The relation between these factors is expressed quantitatively as follows. The mechanism for catalase action is generally represented:

$$\underset{(e-p)}{E} + \underset{(x)}{S} \overset{k_1}{\rightleftharpoons} \underset{(p)}{ES}, \tag{2}$$

$$\underset{(p)}{ES} + \underset{(x)}{S} \overset{k_4}{\rightarrow} E + Q. \tag{3}$$

The lower-case letters under the symbols of equations (2) and (3) represent their concentrations at any time t. The kinetic equations are:

$$\frac{dp}{dt} = k_1 x(e-p) - k_4 xp, \tag{4}$$

$$\frac{dx}{dt} = -k_1 x(e-p) - k_4 xp. \tag{5}$$

In the steady state $dp/dt = 0$, and equation (4) is solved for p/e:

$$\frac{p}{e} = \frac{1}{1 + k_4/k_1}. \tag{6}$$

This quantity (p/e) is the fraction of the enzyme bound as enzyme-substrate complex, and is 1·0/4·0 for erythrocyte catalase and 1·6/4·0 for bacterial catalase. Equation (6) shows that the quantity p/e is independent of x but dependent upon k_4/k_1. By substitution of these values of p/e, equation (6) also shows that the ratio of the values of k_4/k_1 for erythrocyte and bacterial catalases must be 3·0:1·5. Assuming that the values of k_4 are the same for bacterial and erythrocyte catalases, then the ratio of the values of k_1 is 2:1, in agreement with the experimentally determined ratio ($6 \times 10^7 : 3 \times 10^7$).

The effect of p/e upon the activity of the two catalases as measured in terms of the rate of disappearance of hydrogen peroxide (dx/dt) is shown by adding equations (4) and (5)

$$\frac{dp}{dt} + \frac{dx}{dt} = -2k_4xp. \qquad (7)$$

For the steady state $dp/dt = 0$ and

$$\frac{dx}{dt} = -2k_4xp. \qquad (8)$$

The velocity of disappearance of hydrogen peroxide is directly proportional to p, the molar amount of catalase combined as enzyme-substrate complex. Since in the previous discussion it is assumed that k_4 is constant, and the value of p is found experimentally to be 1·6 times as great for bacterial catalase as for erythrocyte catalase, the relative values of their activities (dx/dt), computed as Kat. f. or k_1' (equation 1) would be expected to differ by a factor of 1·6, as indeed they very nearly do $\left(\frac{5{\cdot}3 \times 10^7}{3{\cdot}5 \times 10^7} = 1{\cdot}5\right)$. Thus the consecutive reaction mechanism affords a satisfactory basis for the observed differences of the erythrocyte and bacterial catalase kinetics. However, we are not yet able to obtain satisfactory agreement between the absolute values of p/e, k_1, k_4 and k_1' on the basis of the simple mechanism.

It is of considerable importance in the theoretical analysis of catalase action to discover a catalase with significantly more than one hydrogen peroxide molecule bound per catalase molecule. Bacterial catalase, with 1·6 hydrogen peroxide molecules so bound, provides direct evidence against a particular theory of catalase action and substantially eliminates it from consideration. This theory postulates that catalases, on combination with one molecule of hydrogen peroxide, acquire 'special properties' which prevent the combination of further molecules of hydrogen peroxide (Chance, 1949*e*). This theory actually fits most of the data on erythrocyte catalase, but may now be rejected since with bacterial catalase 1·6 molecules of hydrogen peroxide are bound. The alternative theory that the binding of hydrogen peroxide molecules is purely a statistical effect in the consecutive reactions of hydrogen peroxide with free catalase and catalase-hydrogen peroxide is therefore retained as the most promising explanation for catalase action.

Whereas bacterial catalase is relatively more active than erythrocyte catalase towards hydrogen peroxide, it is relatively less active with larger substrate and acceptor molecules. The decrease in the reactivity of erythrocyte catalase towards substrates and acceptors of increasing size has already been commented upon (Chance, 1949*d*). However, the effect is much more striking with bacterial catalase. The velocity constants for its reaction with methyl and ethyl hydrogen peroxide are each about 10 times smaller than the values for erythrocyte catalase. And in the reactions with acceptors, nitrite reacts more rapidly, while propanol, which reacts at an appreciable rate with erythrocyte catalase-peroxides, gives a scarcely measurable rate

Table 2. *A comparison of the velocity constants for the reactions of bacterial and erythrocyte catalases with substrates and acceptors*

(Velocity constants for the combination of enzyme and substrate. Units M^{-1} sec.$^{-1}$)

Substrate	Hydrogen peroxide	Methyl hydrogen peroxide	Ethyl hydrogen peroxide
Erythrocyte catalase	3×10^7	8×10^5	20×10^3
Bacterial catalase	6×10^7	$2{\cdot}2 \times 10^5$	$2{\cdot}8 \times 10^3$

(Velocity constants for the reaction of the primary enzyme-substrate complexes with acceptors. Units M^{-1} sec.$^{-1}$)

Acceptor	Nitrite*	Formaldehyde	Methanol	Ethanol	Formate*	Propanol
Erythrocyte catalase-hydrogen peroxide, I	2000	—	1000	1000	470	17
Bacterial catalase-hydrogen peroxide, I	2800	—	91	13	175	0·025
Erythrocyte catalase-methyl hydrogen peroxide, I	—	1400	910	910	460	—
Bacterial catalase-methyl hydrogen peroxide, I	—	270	54	11	150	—

* pH = 7·0.

with bacterial catalase-peroxides. These various velocity constants are summarized in Table 2 and amplify the comparison considerably. Reasoning along the same lines as with erythrocyte catalase, it is concluded that the haematins of bacterial catalase are even less accessible than those of erythrocyte catalase, and only very small molecules are suitable as acceptors. Following this idea further, it is proposed that the greater proximity of protein and prosthetic group of bacterial catalase causes interactions which result in a higher activity towards hydrogen peroxide. In other words, the proximity of protein and prosthetic group in bacterial catalase enhances its activity towards small molecules but results in steric effects which abruptly decrease the activity towards larger molecules. Thus rather indirect evidence is presented for the spatial interaction of protein and prosthetic group resulting in increased activity and a higher specificity of the enzyme. Further studies of related haemoproteins are required to extend this theory.

SUMMARY

1. Bacterial catalase forms primary and secondary enzyme-substrate complexes with hydrogen peroxide, methyl hydrogen peroxide and ethyl hydrogen peroxide.

2. The spectra of the bacterial and erythrocyte catalase-methyl hydrogen peroxide complexes in the region of the Soret band are nearly identical. However, a quantitative difference exists between the Soret bands of the primary hydrogen peroxide complex of bacterial and erythrocyte catalases (see below).

3. The velocity constant* for the decomposition of hydrogen peroxide in the presence of bacterial catalase is $5{\cdot}3 \times 10^7$ M^{-1} sec.$^{-1}$ for a four-haematin catalase, and may be compared with the value of $3{\cdot}5 \times 10^7$ for erythrocyte catalases; i.e. the ratio of the velocity constants (or of the Kat. f. values) for the two enzymes is 1·5 : 1.

4. The bacterial catalase-hydrogen peroxide complex consists of $1{\cdot}6 \pm 0{\cdot}1$ hydrogen peroxide molecules per bacterial catalase molecule, as determined by spectrophotometric measurements of catalase-methyl hydrogen peroxide and cyanide compounds. This value may be compared with that of $1{\cdot}0 \pm 0{\cdot}1$ found for erythrocyte catalase on the basis of methyl hydrogen peroxide data.

5. The velocity constant for the combination of bacterial catalase with hydrogen peroxide is about 6×10^7 M^{-1} sec.$^{-1}$ and the reaction is shown to be of the second order up to 20 μM-hydrogen peroxide. This value may be compared with that of about 3×10^7 for erythrocyte catalase.

6. On the basis of the mechanism for catalase action which involves consecutive reactions of hydrogen peroxide with catalase and with the catalase-hydrogen peroxide complex, the greater activity of bacterial catalase compared with erythrocyte catalase may be quantitatively attributed to the more rapid combination of bacterial catalase with hydrogen peroxide to form the bacterial catalase-hydrogen peroxide complex. The consequence of this more rapid reaction is a larger steady-state concentration of the bacterial catalase-hydrogen peroxide complex and a higher velocity constant for the disappearance of hydrogen peroxide.

7. The velocity constants for the combination of bacterial catalase with methyl or ethyl hydrogen peroxide are $2{\cdot}2 \times 10^5$ and $2{\cdot}8 \times 10^3$ M^{-1} sec.$^{-1}$ respectively. These values are about 10 times *slower* than the corresponding values for erythrocyte catalases.

8. The primary bacterial catalase-peroxide complexes react with acceptors according to the mechanisms found to obtain for the corresponding erythrocyte catalase-peroxide complexes; only the green primary complexes appear to be involved in these reactions. The specificity appears to follow an identical pattern; velocity constants are evaluated for nitrite, formaldehyde, formate, methanol, ethanol and propanol.

9. Bacterial catalase and hydrogen peroxide oxidizes nitrite more rapidly than does erythrocyte catalase. Also bacterial catalase is relatively more active towards formate and formaldehyde with respect to methanol and ethanol than is erythrocyte catalase.

10. In the reactions of bacterial catalase with substrates or acceptors, the values of the reaction velocity constants are dependent on the size of the substrate or acceptor molecule to a much greater extent than was found to be the case with erythrocyte catalase; large molecules react much more slowly than small ones. This greater dependence is interpreted to indicate that the haematins of bacterial catalase are 'buried' in the protein molecule to a greater extent than are the haematins of erythrocyte catalase. Nevertheless, the haematins of bacterial catalase react with hydrogen peroxide more rapidly than do the haematins of erythrocyte catalase. It is suggested that the proximity of the protein groups to the haematins of bacterial catalase enhances their activity towards molecules to which the haematins are accessible.

* Velocity constants are measured at 20–25°.

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The Site of Hydrochloric Acid Production in the Stomach as Determined by Indicators*

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In order to correlate the biochemical function and the morphological structure of acid-secreting gastric mucosa, it is necessary to know exactly where the hydrochloric acid is formed. Bernard (1858) first used microchemical methods in an attempt to locate the site of formation of the hydrochloric acid, and Babkin (1944) reviewed the work on this topic. Although it is generally held that the oxyntic cells secrete hydrochloric acid at concentrations about isotonic with blood (Hollander, 1943), some workers (Hoerr & Bensley, 1936) have stated that a precursor of the acid occurs within the gastric tubules and is hydrolysed to form hydrochloric acid in the foveolae or even the lumen of the stomach.

Frog gastric mucosa can secrete some dyestuffs *in vivo* (Henning, 1932) and hydrochloric acid *in vitro* (Delrue, 1930). Tied bags of isolated frog gastric mucosa were used in this study to find out which dyestuffs are transported across the tissue without interfering with respiration or acid secretion. Observations were made of dye- and acid-secreting oxyntic cells in both amphibian and mammalian gastric mucosa. These established the fact that the contents of the canaliculae of these cells have a pH $< 1{\cdot}4$. This shows that acid is formed in the pericanalicular zone of oxyntic cells and is incompatible with the hypothesis that some hypothetical precursor of the hydrochloric acid is secreted by oxyntic cells.

* A part of this work was communicated to the Biochemical Society (Bradford & Davies, 1948).

EXPERIMENTAL

Saline media. Mammalian tissue was incubated in the physiological salt solution (bicarbonate saline) of Krebs & Henseleit (1932), gassed with 5% CO_2 + 95% O_2. For experiments on frog gastric mucosa this solution was diluted with 0·29 vol. of water, when the balance of the O_2 uptake and the CO_2 output was to be measured. The phosphate saline of Krebs (1933) diluted with 0·29 vol. of water was used for measurements of the O_2 uptake of frog gastric mucosa.

The solution used during the preparation of the mucosa was a basal saline similar to amphibian bicarbonate saline but had a HCO_3^- content of 0·004 M and, under working conditions, pH 7·4. All the salines contained 0·2% (w/v) glucose, and histamine was added at the start of all experiments to a final concentration of 5×10^{-5} M.

Dyestuffs. The following dyestuffs were used. *Basic dyes*: acridine, acriflavine, azur I, azur II, benzene-azo-α-naphthylamine, Bismarck brown, brilliant cresyl blue, brilliant green, chrysoidin, dimethyl yellow, malachite green, methyl violet R.S., methylene blue, neutral red, rhodamine *B*, rhodamine-6*G*, safranine-*O*, thionine, toluene-azo-aminotoluene 2:1:1′:4′:3′ and toluidine blue. *Acid dyes*: bromthymol blue, congo red, cresol red, fluorescein, methyl orange and phenolphthalein.

Material. Frogs (*Rana temporaria temporaria* L.) and toads (*Bufo bufo bufo* L.) were captured locally and housed in an outdoor froggery under natural conditions. Six-month old polecats and cats were obtained from a local breeder.

Preparation of the tissue. Frog and toad gastric mucosa was washed, isolated and closed with silk thread at both ends according to the method of Davies (1948*a*). In all cases the pyloric antrum was completely removed and the

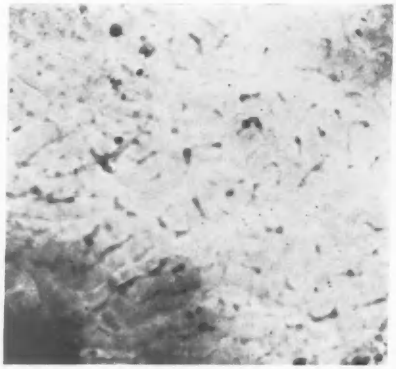

Fig. 1.

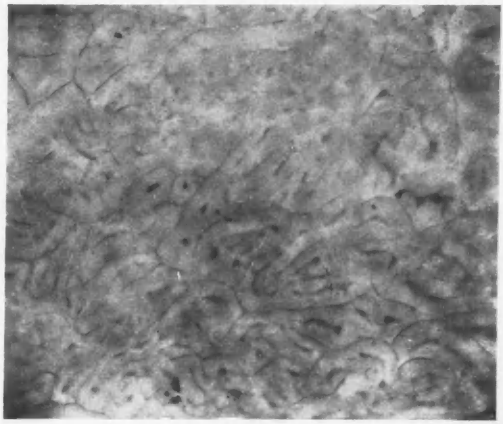

Fig. 2.

Fig. 1. Living, isolated, toad gastric mucosa secreting toluene-azo-amino-toluene 2:1:1′:4′:3′. View downwards on to the secretory surface. The dyestuff in the gastric crypts could be followed down into the tubules (× 50).

Fig. 2. Living, isolated, frog gastric mucosa secreting toluene-azo-amino-toluene 2:1:1′:4′:3′. View downwards on to the secretory surface. Not all the tubules were secreting the dyestuff. Some regions of the mucosa were resting (× 50).

Fig. 3.

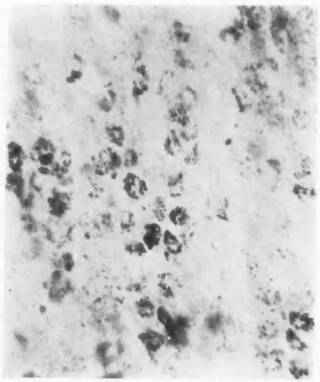

Fig. 4.

Fig. 3. Living, isolated, frog gastric mucosa secreting neutral red. Section of tissue and view through the muscularis mucosae. Many of the dots visible in the lower half of the photo are the gastric tubules, seen end on through the muscularis mucosae, filled with crimson neutral red. The rest of the tissue was yellowish. The cut edge at the top has curled over so that the tubules are visible from the side (× 60).

Fig. 4. Living, isolated tubules from polecat gastric mucosa secreting neutral red. The canaliculi of the oxyntic cells contained neutral red in the acid form. Slight alterations of the focus showed the 'basketwork' of canaliculi connected together in the cells (× 330).

tied bag of mucosa made from the corpus of the gastric tissue. The polecats and cats were killed by a blow on the head. The stomach was removed at once, opened and washed in ice-cold Krebs phosphate saline. Mucosa from the corpus was freed from the muscle layers and used directly.

Manometric procedure. The O_2 uptake and the resultant of O_2 uptake and CO_2 output were measured with Warburg manometers in the usual way. Conical cups (20–30 ml. capacity) with a centre well were used. Dyestuffs were added from the side-arm as crystals or in solution as required.

Technique for the observation of tissues

Foveolae and mucous cells. The tied bag of secreting frog or toad gastric mucosa was removed from the Warburg cup, cut open and the contents removed. The silk was cut away and the sheet of tissue laid, nutrient side downwards, in a shallow pool of basal saline, containing 5×10^{-5}M-histamine, on a microscope slide. The mucosa is so thin that illumination from below was satisfactory for the use of the two-thirds objective. It was found that if both sides of the tissue were immersed in the basal saline, the dyes were soon washed out of the gastric foveolae.

Gastric tubules and oxyntic cells. The mucosa was placed, secretory side downwards, on a microscope slide. The slight contraction of the muscularis mucosae then caused the cut edges of the tissue to curl up slightly so that some of the tubules lay in a horizontal plane and could be observed easily. The muscularis mucosae is so transparent that the secreting oxyntic cells could also be watched.

Isolated oxyntic cells. The mucosa was examined by the technique described in the previous paragraph and a region containing many actively secreting oxyntic cells was selected. This region was cut out with very fine scissors and the tiny piece of tissue placed in a drop of basal saline and teased apart with fine needles held in a semi-micro manipulator (Harding, 1939). A coverslip was placed over the drop and pushed down gently to separate the debris. Most preparations contained a few isolated, secreting oxyntic cells which could be observed for a few minutes with a one-sixth objective until they presumably became anoxic due to the lack of fresh supplies of oxygen under the coverslip.

RESULTS

Secretion of basic dyes. The results of the seventy-five experiments with tied bags of frog gastric mucosa are given in Table 1.

It is clear that the isolated tissue is able to transport a variety of dyestuffs from the nutrient medium to the lumen of the stomach. All the basic (chromogen in cation) dyes were found to accumulate in the secretions from 10^{-3}M or saturated solution whichever was weaker. Though quantitative estimations were not made in all cases, it was found that many of the basic dyes were concentrated in the secretions up to twenty times or more. The acid dyes only appeared in smaller concentrations than those in the saline solutions.

The site of formation of the acid. Some of the basic dyes are acid-base indicators and were used to locate the site of formation of the hydrochloric acid. Neutral red proved especially suitable as it forms solutions in acid which are highly coloured and non-toxic. By microscopical observation of the mucosa it was possible to see that the gastric tubules and the canaliculi of secreting oxyntic cells were red with the acid form of the dye, whilst the cytoplasm of the oxyntic cells and the lower regions of the mucosa were yellowish and slightly alkaline (Pl. 2, Fig. 3). This is in accordance with the fact that these cells secrete as many bicarbonate as hydrogen ions (Davies, 1948*a*). An increase in the pH of the cytoplasm of oxyntic cells with secretion is expected (Davies & Roughton, 1948).

In all mucosae so far examined the activity occurred in patches. Within these patches some glands were quiescent, others actively secreting, and within the active glands some oxyntic cells were not secreting the dyestuff.

The pH *of the secreted acid.* The experiments with neutral red showed that the acid is formed with a $pH < 6{\cdot}8$ (Kolthoff, 1932). This is not very impressive; acridine was therefore used which has a green fluorescence at a pH below 4·85 and a pale blue one above this pH (Kolthoff, 1932). Care is needed to interpret the microscopic picture since filled blood vessels in the mucosa have a greenish colour in ultraviolet light, but the experiments showed that the newly formed acid has a $pH < 4{\cdot}85$.

The best evidence was obtained with toluene-azo-amino-toluene 2:1:1′:4′:3′. This is red at a $pH < 1{\cdot}4$ (Kolthoff, 1932), but the free base is very insoluble, and successful results were only obtained by adding the dye in freshly precipitated microcrystalline form obtained by rapid neutralization of a solution of the hydrochloride. Although the red colour could be seen easily in the foveolae and tubules (Pl. 2, figs. 1, 2) this dye has such a low tinctorial value that the colour in the tiny canaliculi, though visible by eye, was too faint for photography. Again care is needed to distinguish occasional blood vessels from tubules full of red dye.

The technique of providing an excess of a very insoluble dyestuff in the form of microcrystals in the nutrient medium, makes it possible to study the secretion of such dyes. Microcrystals are more soluble than coarse crystals because of their higher surface activity. When dye is removed from the nutrient medium by the mucosa, more of the crystals dissolve, thus maintaining the concentration in the medium. In the intact animal such experiments might be impossible because the very small amount of dye in solution in the blood could not be replaced. Injected crystals would block up capillaries and would thus be largely removed from the circulation. The concentration of dye in the blood would then probably fall too low for the dye to be seen in the gastric juice.

Table 1. *Secretion of acid and basic dyes by tied bags of frog gastric mucosa*

Dye	Exp. no.	Chemical class	Conc. in medium (M)	Occurrence in secretion	Secretions acid (*A*), secretions neutral or alkaline (*B*)	Average Q_{HCl} (μl./mg. dry wt./hr.)	Average Q_{O_2} (μl./mg. dry wt./hr.)	Time in presence of dyestuff (hr.)	Remarks
Basic dyes									
Acridine	1	Acridine	Saturated	+	*A*	2·2	—	0·5	Fresh microcrystals
Acridine	2	Acridine	Saturated	+	*A*	0·7	—	2·5	Fresh microcrystals
Acriflavine	3	Acridine	10^{-3}	+	*A*	0·1	—	2·0	—
Acriflavne	4	Acridine	10^{-4}	+	*A*	4·0	—	1·0	—
Azur I	5	Thiazine	10^{-3}	+	*A*	3·5	—	2·5	—
Azur II	6	Thiazine	10^{-3}	+	*A*	3·7	—	2·5	—
Benzene-azo-α-naphthylamine	7	Azo	Saturated	+	*A*	—	−1·1	1·5	—
Benzene-azo-α-naphthylamine	8	Azo	Saturated	+	*A*	2·1	—	1·5	—
Benzene-azo-α-naphthylamine	9	Azo	Saturated	+	*A*	—	−1·8	1·5	—
Benzene-azo-α-naphthylamine	10	Azo	Saturated	−	*B*	0	—	1·5	—
Bismarck brown	11	Bisazo	Saturated	+	*A*	7·1	—	2·5	—
Brilliant cresyl blue	12	Oxazine	10^{-3}	+	*A*	2·3	—	2·5	—
Brilliant cresyl blue	13	Oxazine	10^{-3}	+	*A*	2·9	—	2·5	—
Brilliant cresyl blue	14	Oxazine	10^{-3}	+	*A*	—	−2·25 −3·6	3·0	Large increase in respiration
Brilliant cresyl blue	15	Oxazine	10^{-4}	+	*A*	—	−3·1 −5·9	2·0	Large increase in respiration
Brilliant cresyl blue	16	Oxazine	10^{-4}	+	*A*	—	−3·5 −4·5	2·0	Large increase in respiration
Brilliant cresyl blue	17	Oxazine	10^{-4}	+	*A*	1·3	—	2·5	Acid secretions not increased
Brilliant green	18	Triphenylmethane	10^{-3}	+	*A*	1·9	—	2·0	Acid secretions not increased
Brilliant green	19	Triphenylmethane	10^{-4}	+	*B*	0	—	2·0	Acid secretions not increased
Chrysoidin	20	Azo	Saturated	+	*A*	—	−1·5	2·5	Slight inhibition
Chrysoidin	21	Azo	Saturated	+	*B*	0	−1·9	2·5	Slight inhibition
Dimethyl yellow	22	Azo	Saturated	+	*A*	0·2	—	3·0	—
Dimethyl yellow	23	Azo	Saturated	−	*B*	0	—	2·0	—
Malachite green	24	Triphenylmethane	10^{-3}	+	*A*	1·2	—	2·0	Secretion stopped after 1 hr.
Methyl violet R.S.	25	Triphenylmethane	10^{-3}	Trace	*A*	1·2	—	2·5	—
Methyl violet R.S.	26	Triphenylmethane	10^{-3}	Trace	*A*	—	−1·9	1·5	—
Methyl violet R.S.	27	Triphenylmethane	10^{-5}	−	*B*	0	—	2·0	—
Methylene blue	28	Thiazine	10^{-3}	+	*A*	0·1	—	2·5	—
Methylene blue	29	Thiazine	10^{-4}	+	*A*	5·0	—	1·0	—
Methylene blue	30	Thiazine	10^{-4}	+	*B*	0	—	1·0	—
Methylene blue	31	Thiazine	10^{-4}	+	*A*	—	−3·8	2·0	—
Neutral red	32	Azine	10^{-3}	+	*A*	4·0	—	1·5	—
Neutral red	33	Azine	10^{-3}	+	*A*	—	−2·1	1·5	—
Neutral red	34	Azine	10^{-3}	+	*A*	3·6	—	2·0	—
Neutral red	35	Azine	10^{-3}	+	*A*	2·2	—	1·5	—

Neutral red	36	Azine	10^{-3}	–	*B*	0	—	1·0	—
Neutral red	37	Azine	2×10^{-4}	+	*A*	1·2	—	1·5	—
Neutral red	38	Azine	10^{-4}	+	*A*	—	2·7	3·0	—
Neutral red	39	Azine	10^{-4}	+	*A*	0·5	—	2·5	—
Neutral red	40	Azine	10^{-4}	+	*A*	6·5	—	2·0	—
Neutral red	41	Azine	10^{-4}	+	*A*	5·8	—	2·0	—
Neutral red	42	Azine	10^{-5}	+	*A*	—	2·8	3·0	—
Neutral red	43	Azine	10^{-5}	+	*A*	2·1	—	2·0	—
Neutral red	44	Azine	10^{-6}	+	*A*	—	2·5	3·0	—
Neutral red	45	Azine	10^{-6}	+	*A*	4·4	—	2·0	—
Neutral red	46	Azine	10^{-7}	–	*A*	—	3·3	3·0	—
Neutral red	47	Azine	10^{-7}	–	*A*	3·2	—	2·0	—
Neutral red	48	Azine	10^{-8}	–	*A*	—	2·1	3·0	—
Neutral red	49	Azine	10^{-9}	–	*A*	4·5	—	2·0	—
Rhodamine-*B*	50	Pyronine	10^{-3}	+	*A*	0·3	—	2·0	—
Rhodamine-6*G*	51	Pyronine	10^{-3}	+	*A*	2·9	—	2·5	—
Safranine 0	52	Azine	10^{-3}	+	*A*	0·5	—	2·5	—
Thionine	53	Thiazine	10^{-3}	+	*A*	0·1	—	2·0	—
Thionine	54	Thiazine	10^{-3}	+	*B*	0	2·6	4·0	—
Thionine	55	Thiazine	10^{-3}	+	*A*	0·7	—	3·0	—
Toluene-azo-amino-toluene 2:1:1′:4′:3′	56	Azo	Saturated	–	*A*	1·8	—	1·0	—
Toluene-azo-amino-toluene 2:1:1′:4′:3′	57	Azo	Saturated	+	*A*	1·7	—	2·0	Fresh microcrystals
Toluene-azo-amino-toluene 2:1:1′:4′:3′	58	Azo	Saturated	+	*A*	—	3·2	1·5	Fresh microcrystals
Toluene-azo-amino-toluene 2:1:1′:4′:3′	59	Azo	Saturated	+	*A*	0·7	—	1·5	Fresh microcrystals
Toluene-azo-amino-toluene 2:1:1′:4′:3′	60	Azo	Saturated	+	*A*	3·0	—	2·0	Fresh microcrystals
Toluene-azo-amino-toluene 2:1:1′:4′:3′	61	Azo	Saturated	+	*A*	1·7	—	1·5	Fresh microcrystals
Toluene-azo-amino-toluene 2:1:1′:4′:3′	62	Azo	Saturated	–	*B*	0	—	1·0	Fresh microcrystals
Toluene-azo-amino-toluene 2:1:1′:4′:3′	63	Azo	Saturated	+	*A*	3	—	2·0	Fresh microcrystals
Toluene-azo-amino-toluene 2:1:1′:4′:3′	64	Azo	Saturated	+	*A*	2	—	2·5	Fresh microcrystals
Toluidine blue	65	Thiazine	10^{-3}	+	*B*	0	—	2·5	—
Acid dyes									
Bromthymol blue	66	Pyronine	10^{-3}	Trace	*A*	1·8	—	1·5	—
Bromthymol blue	67	Pyronine	10^{-4}	Trace	*B*	0	—	2·0	—
Congo red	68	Azo	10^{-3}	–	*A*	0·4	—	2·5	—
Cresol red	69	Pyronine	10^{-3}	+	*B*	0	—	2·0	—
Cresol red	70	Pyronine	10^{-3}	Trace	*A*	—	1·5	2·5	—
Cresol red	71	Pyronine	10^{-4}	Trace	*A*	0·6	—	3·5	—
Fluorescein	72	Pyronine	10^{-4}	–	*A*	4·0	—	1·0	—
Methyl orange	73	Azo	10^{-3}	Trace	*A*	0·1	—	4·0	—
Methyl orange	74	Azo	10^{-4}	Trace	*A*	3·2	—	1·5	—
Phenolphthalein	75	Pyronine	10^{-3}	–	*A*	3·4	—	2·5	—

Experiments with mammalian mucosa. With due precautions the same technique can be applied to mammalian mucosa. Most types of mammalian gastric mucosa are too thick to be effectively oxygenated *in vitro* (Davies, 1946, 1948*b*; Davenport & Jensen, 1948; Patterson & Stetten, 1949) but the relatively thin gastric mucosa of the long thin polecat (*Putorius putorius*) can be used for the study of the *in vitro* secretion of acid (Davies, 1948*b*). It was soon found that the secretion of dyestuffs could also be observed with cat gastric mucosa if the material was cut into small pieces.

Samples of the gastric mucosa from these animals were cut into small pieces with fine scissors and incubated at 37° in solutions containing 5×10^{-5}M-histamine, or 5×10^{-5}M-carbamyl-choline chloride, together with neutral red (Pl. 2, fig. 4), acridine or toluene-azo-amino-toluene 2:1:1′:4′:3′. In these species too, the contents of the intracellular canaliculi of some of the oxyntic cells had a pH < 1·4. In both amphibian and mammalian oxyntic cells, neutral red sometimes accumulated within the cytoplasm as red spherical granules.

Methyl violet R.S., which changes colour from pH 0·1–3·2 (Kolthoff, 1932) might be expected to give useful information, but unfortunately it was secreted in such tiny amounts by frog, toad, cat and polecat mucosa that no conclusive results were obtained under the experimental conditions used.

Secretion of acid dyes. The acid (chromogen in anion) dyes, congo red and phenolphthalein, were not transported, but bromthymol blue, cresol red, fluorescein and methyl orange were sometimes found in traces inside the mucosal bags especially if their secretions were alkaline or only slightly acid.

DISCUSSION

The site of formation of the hydrochloric acid. Although in the last 90 years more than thirty workers have tried to locate the site of formation of the hydrochloric acid produced by gastric mucosa, Babkin (1944) wrote 'Unfortunately as yet none of the histological, microchemical or physiological investigations of the problem permit one to draw any definite conclusions on the subject'.

Bernard (1858) injected 'lactate de fer', which Sehrwald (1889) claimed was largely ferrous, not ferric, lactate, and potassium ferrocyanide into a rabbit which was killed soon after. A deposit of prussian blue was found only on the surface of the gastric mucosa and none could be detected microscopically in the tubules. He concluded that the acidity of the gastric juice only appeared after the secretion from the tubules had reached the surface and had become mixed with the fluids in the stomach.

Brücke (1859), however, believed that the acid was produced free in the gastric tubules. He pressed rabbit gastric mucosa between litmus paper and found that whilst the free surface was acid, the deeper portions were alkaline. He concluded that if a free acid was liberated in the gastric tubules, an alkaline fluid of equivalent strength must at the same time pass into the blood and lymph systems. Lepine (1872) confirmed Bernard's results and unsuccessfully attempted to demonstrate the presence of acid using macerated tissue. Edinger (1882) used the indicators tropaeolin, phenolphthalein and Na alizarin. He was unable to find out where the HCl was formed but showed that only small areas of the mucosa were in full secretory activity at any one time. This effect has been confirmed during the experiments described in this paper.

Similar negative results with dyestuffs, under conditions such that the gastric mucosa could not have been secreting when the microscopical observations were made, were recorded by Trinkler (1884), Stintzing (1889), Sehrwald (1889) and Fränkel (1891).

The next major attack on the problem depended largely on the prussian blue reaction. Macallum (1908), Fitzgerald (1910), Hammett (1915) and Collip (1920) claimed to have found occasional deposits of prussian blue, and hence of free HCl, in the gastric tubules and even in the intracellular canaliculi. Harvey & Bensley (1912) criticized Fitzgerald's (1910) conclusions because they found prussian blue deposits in many other places in the body and believed, with Bernard (1858), that the HCl was formed on the surface of the gastric mucosa from some precursor. They in turn were severely criticized by Collip (1920) for using very toxic solutions (25% ammonium ferric citrate and 10% sodium ferrocyanide), which were so concentrated that they precipitated prussian blue when mixed, even in the absence of any acid.

Chambers (1915) injected neutral red into the oxyntic cells of a freshly killed mouse and wrote: 'the results seem to leave no room for doubt that the immediate secretion of the parietal cell with respect to neutral red is neutral if not actually alkaline'. However, his experimental technique was such that the cells he was observing could not have been secreting. Dawson & Ivy (1926) found that a blow on the head of a Pavlov-pouch dog, which lived for a further 40 min., completely stopped the secretion of HCl in response to 2 mg. of histamine given 7 min. before, and no secretory effect was observed following the injection of 2 mg. of histamine 25 min. after the blow. They also showed that the oxyntic cells of freshly killed mammals do not secrete. This has been confirmed by finding that a recovery period is required in both amphibian and mammalian isolated gastric mucosa before acid secretion begins (Davies, 1948*a*, *b*).

Further inconclusive experiments on non-acid-secreting mucosa were published by Glässner, Wittgenstein & Hamperl (1925), Zimmermann (1925), Linderstrøm-Lang, Holter & Ohlsen (1935) and Ostrouch (1937). The work of Linderstrøm-Lang *et al.* (1935) is of special importance. They cut serial sections of cylinders of pig gastric mucosa parallel to the surface and titrated them for acid. They found a correlation between the number of oxyntic cells and the acid in each slice and calculated the normality of the whole volume of these cells to be 0·15N with respect to acid. This work has been quoted by Hollander (1943) as done on 'actively secreting fundus mucosa'. Linderstrøm-Lang *et al.* (1935) said the experiments were done on mucosa removed from the pig several hours earlier and mentioned the 'many unsuccessful attempts to estimate acid in fresh gastric mucosa'. The observations are unexpected and the explanation is probably that given by Linderstrøm-Lang (1947):

'We are not quite certain about the origin of the acid found in our mucosas, but I think that an invasion of HCl from the contents of the stomach is the main cause.'

Hoerr & Bensley (1936) used a new technique of freezing the fresh mucosa in liquid pentane, air or nitrogen and drying under vacuum. They were unable to detect HCl subsequently in the tubules. However, with this technique any free HCl would be removed along with the water in the tissue. Gersh (1938) also freeze-dried gastric mucosa and found that 'chloride was absent from the cytoplasm of all gland cells' and that 'the distribution of chloride in the actively secreting mucous membrane did not differ essentially from that in the resting tissue'. Since he killed his animals by bleeding under ether anaesthesia it is certain that the mucosa was not actively secreting when frozen. His conclusions are in contradiction to those of Collip (1920) and Hogartz (1932) who found large increases in the chloride content of gastric mucosa during secretion.

Thus the previous work on isolated non-secreting mucosa has not solved the problem of the site of formation of the hydrochloric acid.

Henning (1932) observed the secretion of some dyes by living narcotized frogs with the stomach laid open. He said that neutral red only became red in the orifice of the glands on the surface of the mucosa. Keller & Pisha (1947) said they had repeated this but gave no details. This difference from our results is not easy to explain as insufficient data are available about the conditions in Henning's experiments. He used a white arc light to illuminate the mucosa through the stomach muscles and it is possible that the tissue became too hot and thus stopped secreting.

It is of interest that Teorell & Wersäll (1945), who were investigating the electrical impedance properties of frog gastric mucosa, observed that 'In several cases one could successfully remove the top layer of the mucosa cell layer. In spite of this operation the acid secretion continued with unchanged intensity.' This observation supports the view that the hydrochloric acid is formed as such by the oxyntic cells.

There is much evidence that at high rates of secretion the hydrochloric acid is formed primarily at a concentration isotonic with the blood (Hollander, 1932, 1938; Gilman & Cowgill, 1933; Gray & Bucher, 1941); at lower rates of secretion the primary concentration may be even higher than this (Linde, Teorell & Öbrink, 1947).

The fact that the intracellular canaliculi contain acid at a $pH < 1{\cdot}4$ during acid secretion shows that the site of formation of the hydrochloric acid is in the pericanalicular zone of the oxyntic cells, and that no precursor is secreted which turns into hydrochloric acid in the foveolae or the lumen of the stomach as believed by Bernard (1858), Ebstein & Grützner (1874), Osborne (1901), Harvey & Bensley (1912), Zimmermann (1925), Hanke (1926), Hanke & Donovan (1927), Hoerr & Bensley (1936) and Ostrouch (1937).

The mechanism of secretion of dyestuffs. Visscher (1942) claimed that 'by invoking the theory that dyes enter and pass through pores of charged membranes in undissociated form it becomes possible to harmonize the information we have on dyestuff permeation with the well established facts regarding permeation of strong electrolytes'. This theory would account for the accumulation of basic dyes since the coloured cation would be 'captured' in the acid secretion. Höber (1945, p. 603) supported this view as applied to the stomach but pointed out (p. 265) that some cells do show active accumulation of dyestuffs.

The experiments described show that the acid dye, phenolphthalein, was not secreted, whilst the basic dyes brilliant cresyl blue, thionine and acriflavine were found to accumulate in the lumen of the stomach. The pK_a of phenolphthalein is 9·7 (Kolthoff, 1932) whilst the pK_{ab} values of brilliant cresyl blue and thionine are greater than 11·0. Thus phenolphthalein is virtually unionized, whilst brilliant cresyl blue and thionine are virtually completely ionized at the pH of the nutrient medium (7·4) or the secretions (usually 1–3). Acriflavine is a quaternary ammonium salt and therefore cannot exist in an uncharged form at any physiological pH, it also has a pK_{ab} of 12·0 and is not reduced in living tissue to a substance of lower basicity.

It therefore seems that some charged molecules can pass cell membranes whilst others are held back. The great variety of chemical types of basic dyestuffs that are secreted suggests that the most important factor in determining whether they are secreted is their possible existence as positively charged ions. It may be that they are secreted across the canalicular walls by a tertiary process of the type described by Davies & Ogston (1950).

The transport of acid dyes is probably carried out by the mucous or neck cells along with their bicarbonate-containing secretions. These acid dyes were never observed in the living oxyntic cells although they stain the dead cells. The view that the acid dyes are transported in the stomach by cells elaborating alkaline juices is supported by the observation that acid dyes are secreted by pancreatic cells, which are stained when dead by basic dyes (Visscher, 1942). Morrison, Gardner & Reeves (1936) and Morrison, Reeves & Gardner (1936) claimed that some acid dyes were secreted by Pavlov-pouch dogs. This claim has been disputed by Ingraham & Visscher (1935), Varco & Visscher (1941) and Vischer (1942) who used histamine-stimulated Pavlov-pouch dogs. The former workers did not state how they stimulated secretion and it may be that the results are not inconsistent if they were observing a mixed secretion whilst Visscher and co-workers were observing only oxyntic cell secretion following the injection of histamine (Babkin, 1944).

SUMMARY

1. Basic dyes of many chemical types were transported across isolated acid-secreting frog and toad gastric mucosa and were concentrated in the secretions.

2. Some of the dyes were acid-base indicators. Microscopical observations showed that during acid secretion the gastric crypts, tubules and intracellular canaliculi had pH values < 6·8 (neutral red), < 4·85 (acridine in ultraviolet light) and < 1·4 (toluene-azo-amino-toluene 2:1:1′:4′:3′). Methyl violet, a basic dye with colour changes at still lower pH values, was not secreted in sufficient amounts to determine the pH of the tubules etc. It is concluded that the site of formation of the hydrochloric acid is the wall of the canaliculi of the oxyntic cells (the pericanalicular zone).

3. In all cases the secretory activity of the tissue occurred in patches, and even within active tubules some oxyntic cells were not secreting the dyes.

4. Some acid dyes were transported, but were not concentrated in the secretions.

5. Similar results were obtained using small pieces of isolated polecat and cat gastric mucosa.

6. The mechanism of secretion of dyestuffs is discussed.

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The Fluorimetric Estimation of N^1-Methylnicotinamide and its Differentiation from Coenzyme I

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N^1-Methylnicotinamide has been shown to be one of the principal excretion products derived from the metabolism of nicotinic acid in man, the dog and the rat (Najjar, Scott & Holt, 1943; Huff & Perlzweig, 1943*a*, *b*; Ellinger & Coulson, 1943). Various methods have been worked out for its estimation employing adsorption on to, and elution from, an active surface, followed by extraction into a butanol phase. The fluorescence in the butanol layer, that is the F_2 fluorescence, has then been measured (Huff & Perlzweig, 1943*c*; Coulson, Ellinger & Holden, 1944; Najjar, 1944). Huff, Perlzweig & Tilden (1945), and Huff & Perlzweig (1947) have published a new method for the estimation of N^1-methylnicotinamide, involving the formation of a fluorescence in the presence of acetone in a single aqueous phase. A fluorescence was produced if coenzymes were similarly treated and this method has been used for their estimation in blood (Levitas, Robinson, Rosen, Huff & Perlzweig, 1947) and animal tissues (Robinson, Levitas, Rosen & Perlzweig, 1947).

The present paper deals with studies of the optimum conditions for the production of fluorescence of some N^1-substituted pyridine compounds. A modified procedure has been devised using methyl ethyl ketone instead of acetone, and employing different blanks. The fluorescence due to N^1-methylnicotinamide has been differentiated from that of the phosphopyridine nucleotides and N^1-methylnicotinuric acid betaine. The interference due to indole has been eliminated.

EXPERIMENTAL†

Principle. N^1-methylnicotinamide (*N*-MN) in aqueous alkaline solution reacts with ketones to produce a greenish blue fluorescence; on acidification the fluorescence changes to blue, and is intensified by heating (Huff *et al.* 1945; Huff, 1947).

Choice of ketone. Several ketones were tested both with water alone and with an aqueous solution of N^1-methylnicotinamide chloride (Table 1). We prefer the methyl ethyl ketone to acetone, because it gives higher fluorimeter readings and is less volatile. The time of alkaline treatment and strength of alkali for optimal fluorescence were found to be the same as described for the acetone method (Huff & Perlzweig, 1947). A 5-min. period was adopted for acid digestion (Table 2). A shorter digestion of 2 min. as recommended in the original acetone procedure was found not to be optimal for the methyl ethyl ketone procedure.

Table 1. *Fluorescence of* N-*MN in the presence of various ketones*

Ketone used	Fluorimeter readings (mm.) Water control	Water + 0·15 μg. of *N*-MN/ml.*
Acetone	1	46·5
Methyl ethyl ketone	1	66·5
Diacetone alcohol	8·5	21

* Final concentration as used for fluorimetric readings.

Table 2. *Effect of time of digestion on final fluorescence of* 0·2 μg. *of* N-*MN/ml.*

Time of digestion (min.)	Fluorimeter readings (mm.) Average of five readings	Standard deviation
2	69	±1·5
4	84	±2·9
6	81	±2·1

Acidity of digestion mixture. The fluorescence in methyl ethyl ketone like that in acetone was only slightly altered between pH 1 and 5. An amount of HCl needed to adjust the solution to pH 2 was used throughout (Table 3).

Table 3. *Effect of* pH *during acid digestion on fluorescence of* 0·2 μg. *of* N-*MN/ml.*

Final pH	1	2	5	7	10
Fluorimeter readings (mm.)	86	88	86	73	11

Choice of a blank. The use of blue fluorescence, especially with urine, as a measure of a particular compound has the danger of unspecificity. A critical examination of possible blanks was therefore undertaken. Blanks may be prepared in the following ways: (1) omitting the ketone and replacing it by water (Huff *et al.* 1945), 'water blank'. (2) The addition of the reactants in a different order so that *N*-MN gives no fluorescence, although the final composition of the reactant mixture is unchanged. The blank procedures 2*a*, *b* and *c*, given in Table 4 ('delayed ketone blank', 'final ketone blank' and 'no NaOH blank'), are of this type. The last is included in this group because the omission of both NaOH and the equivalent of HCl leaves the final composition of the solu-

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† Where not otherwise stated the procedure has been carried out as described in the final method.

tion altered only in its NaCl content. (3) The preliminary removal of *N*-MN from the original material by selective adsorption ('decalso blank'). (4) Huff *et al.* (1945) used charcoal to remove interfering substances. We were unable to secure a suitable type of charcoal which did not also adsorb some of the *N*-MN.

Blanks of types 1 *and* 2 (*Table* 4). The blank fluorescence in each case was determined on three test materials: (i) water, (ii) standard *N*-MN solution, and (iii) urine.

With all four of the blanks the fluorimeter readings with pure *N*-MN were no greater than with water as the test material. However, urine gave higher readings with all the blanks, particularly those containing ketone. The possibility that *N*-MN was causing the higher values for the blanks in the presence of urine, although it did not fluoresce in a simple watery solution, was examined by comparing the fluorescence of blanks for a sample of urine and for the same sample of urine with added *N*-MN (Table 5). Blanks 2*a* and 2*b* showed a significant increase with added *N*-MN and could not therefore be recommended. On the other hand, the remaining blanks showed no increase with *N*-MN added to urine.

In further experiments it was found that a trace of acetone (of the order of concentration found in a diabetic urine) increased the 'water blank' significantly, but did not affect the 'no NaOH blank'. The 'no NaOH blank' was therefore to be preferred.

Blank of type 3. A blank of type 3 which we investigated was the 'decalso blank'. A column of fresh, but not specially activated, decalso (60–80 mesh, Permutit Co. Ltd.) 10 cm. long and 8 mm. in diameter was prepared and washed with 10 ml. of water by suction. The 20 ml. sample of test material was adjusted to pH 5·0 with glacial acetic acid, then sucked through the column and the filtrate collected. It was found that if the total salinity of the solution did not exceed 0·5% KCl, more than 98% of the *N*-MN in the original solution was adsorbed. Thus the filtrate, if treated by the normal procedure parallel with a sample which was not filtered, provided a possible blank.

The blank was found to be applicable to urine (Table 6). This blank fluorescence produced with decalso-filtered urine gave a value similar to that produced with the 'no NaOH blank'. *N*-MN added to the urine before passing through the decalso column was completely adsorbed, as evidenced by the fact that the intensity of the fluorescence was not increased. Addition of *N*-MN to the filtered urine increased the fluorescence to the same extent as it did when added to unfiltered urine. The 'decalso blank' has never been found

Table 4. *Fluorescence of different blanks*

	Operations, in order					Fluorimeter readings (mm.)		
Blanks	1	2	3	4	5	Water control	*N*-MN (0·2 μg./ml.)	Urine (0·1 ml. in 1 ml.)
(1) Water	NaOH	Wait 5 min.	HCl, pH 2·0	Digest 5 min.	—	2·0	1·5	53
(2*a*) Delayed ketone	NaOH	Wait 5 min.	HCl, pH 2·0	Ketone	Digest 5 min.	2·5	2·5	63·5
(2*b*) Final ketone	NaOH	Wait 5 min.	HCl, pH 2·0	Digest 5 min.	Ketone	2·5	2·0	62
(2*c*) No NaOH	HCl, pH 2	Ketone	Digest 5 min.	—	—	2·5	2·5	60·5
Full fluorescence	Ketone	NaOH	Wait 5 min.	HCl, pH 2·0	Digest 5 min.	2·5	97	97

Table 5. *Examination of blanks in the presence of urine*

	Fluorimeter readings (mm.)		
Blanks	Urine, 0·08 ml. in 1 ml.	Urine (0·08 ml.) + *N*-MN, (0·15 μg.) in 1 ml.	Increase due to *N*-MN
(1) Water	70	70	0
(2*a*) Delayed ketone	80·5	86·5	+ 6·5
(2*b*) Final ketone	74·5	80·5	+ 6·0
(2*c*) No NaOH	78	'78	0
Full fluorescence	223·5	261·5	+38

Table 6. *Adsorption of* N-*MN in presence of urine*

	Fluorimeter readings (mm.)			
Material	Without added *N*-MN	With added *N*-MN (0·2 μg./ml.)		Increase with *N*-MN
Urine, 0·025 ml. in 1 ml.	62·5	150·5		+88
		Added before filtration	Added after filtration	
Urine, 0·0125 ml. in 1 ml. (filtered through decalso column)	33	32	—	− 1
		—	125·5	+92·5

to be lower than the 'no NaOH blank', suggesting that none of the non-specific fluorescing substances in urine were adsorbed on the decalso column.

FINAL METHOD

Experience with both rat and human urines has shown that there was very little difference between 'decalso' and 'no NaOH' blanks, and for routine examinations of these materials the simpler 'no NaOH' technique will first be described. Precautions other than those described below were taken according to Huff & Perlzweig (1947).

Final procedure 1 (*'no* NaOH *blank'*)

Reagents required. (1) Methyl ethyl ketone; (2) 5 N-NaOH; (3) 5 N-HCl; (4) 20% KH_2PO_4 solution.

Standard N^1*-methylnicotinamide solution*: (1) *stock standard solution*, 0·5 mg./ml. of N^1-methylnicotinamide ion, made by dissolving 31·35 mg. of N^1-methylnicotinamide chloride in 50 ml. of 0·1 N-HCl; (2) *working standard solution*, 2·5 μg./ml., made by diluting 1·0 ml. of the 'stock standard' to 200 ml. and containing 0·01 N-HCl.

Procedure

(*a*) *Chemical.* Take five 50 ml. conical flasks (*A–E*). Flask *A* is the blank, *B* and *C* are duplicate 'unknowns', *D* and *E* are duplicates containing the 'unknown + added standard'. Dilute the extract or solution of the test material so as to contain approximately 2·5 μg. of *N*-MN/ml. To each flask add 2 ml. of the diluted solution.

To flask *A* add 4 ml. of water, 0·5 ml. of 5 N-HCl and 2 ml. of ketone. Heat for 5 min. on a boiling water bath. Evaporation is minimized by placing in the neck of the flask a simple three-bulb air condenser. Cool rapidly by adding 15 ml. of water, then 4 ml. of KH_2PO_4 solution. Make up to 40 ml. with water. The solution is then ready for reading.

To flasks *B* and *C* add 2 ml. of water; and to *D* and *E* 2 ml. of *N*-MN standard (i.e. 5 μg. of *N*-MN). Then to the four flasks add 2 ml. of ketone and 1 ml. of 5 N-NaOH. Wait exactly 5 min., and then add 1·5 ml. of 5 N-HCl. Heat for 5 min. and proceed as for flask *A*.

(*b*) *Fluorimetric.* A single photo-cell fluorimeter (Cohen type) is used, together with a sensitive galvanometer (3800 mm./μa., with light scale 2 m. from mirror). The filters used are a 'Calorex' and a Wood's glass in the exciting beam of a mercury lamp. For the blue 'thiochrome-like' fluorescence, Wratten filters nos. 2A and 47 are placed in front of the photo cell. Readings are taken in cells of 20 ml. capacity.

Calculation. Let X = galvanometer deflexion for flask *A* (blank); Y = mean of galvanometer deflexions for flasks *B* and *C* (unknown); Z = mean of galvanometer deflexions for flasks *D* and *E* (unknown + 5 μg. of *N*-MN); R = dilution factor of original extract to obtain a suitable sample in 2 ml. of 'unknown'. Then the extract contains

$$\frac{Y-X}{Z-Y}\times\frac{5}{2}\times R\,\mu g.$$

of N^1-methylnicotinamide/ml.

Final procedure 2 (*'decalso blank'*)

In the procedure using the 'decalso blank', the 'no NaOH blank' in flask *A* is replaced by a blank in which the fluorescence of a decalso filtrate is developed exactly as in flasks *B–E*.

Dilute a portion of the extract of the test material so as to contain approximately 2·5 μg. of *N*-MN/ml. For the blank adjust another portion of the extract to pH 5 with glacial acetic acid and make up to the same concentration as above.

Preparation of the blank. Take 20 ml. of the diluted solution adjusted to pH 5. Pass with suction through a column of decalso 60–80 mesh, 10 cm. long, 8 mm. in diameter, which has been previously washed with 10 ml. of water. Collect the filtrate.

Chemical. To flask *A* add 2 ml. of the filtrate, and to flasks *B*, *C*, *D* and *E* 2 ml. of the diluted test material. To flask *A*, *B* and *C* add 2 ml. of water; and to *D* and *E* 2 ml. of *N*-MN standard. Then to all five flasks add 2 ml. of ketone and 1 ml. of 5 N-NaOH. Wait exactly 5 min., and then add 1·5 ml. of 5 N-HCl. Heat for 5 min. on a boiling water bath. Cool rapidly by adding 15 ml. of water, then 4 ml. of KH_2PO_4 solution. Make up to 40 ml. with water. Proceed with the fluorimetric readings and use the same calculations as in Procedure 1.

As a check for the complete adsorption of *N*-MN on the decalso column, take 20 ml. of the diluted test solution containing in addition 50 μg. of added *N*-MN, and proceed with filtration through a decalso column. Add 2 ml. of the filtrate to a sixth flask *F*, and work through the method as for flask *A*. If the fluorescence resulting is greater than in flask *A* the estimation must be repeated with a more dilute test solution so as to reduce its salinity.

Reproducibility of fluorescence

Using the results from thirty urine estimations, the standard deviation of the differences between the readings of duplicate flasks *B* and *C* was calculated, and found to be ±2·9 mm. The mean of all the readings was 66·7 mm., so that the standard deviation for a single reading at the usual level of *N*-MN may be expressed as ±4·4% and the standard error of duplicate flasks as $\pm\frac{4\cdot4}{\sqrt{2}}=\pm3\cdot1\%$.

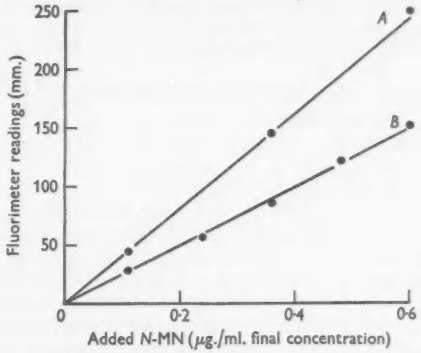


Fig. 1. Relation between concentration of *N*-MN and fluorescence, in aqueous medium and in the presence of urine; curve *A*, in water; curve *B*, in urine, diluted 1:8.

Test of straight-line relationship

The calculation described above does not depend upon a standard reference curve, but implied in it is a straight-line relation between concentration of *N*-MN and the galvanometer reading. This has been tested both in pure solution and in urine (Fig. 1).

The results establish a straight-line relationship within the limits of the concentrations used for both urine and an aqueous solution of *N*-MN. It will be seen that the fluorescence of a given quantity of *N*-MN is less in the presence of urine. This emphasizes the importance of an internal standard (Huff & Perlzweig, 1947).

Specificity

Various substances were tested to see if they might interfere in the method. The following pyridine derivatives gave no fluorimeter reading in amounts of 20–80 μg.: nicotinic acid, nicotinic amide, nicotinic acid diethyl amide, trigonelline, nipecotic acid, picolinic acid, quinolinic acid, aminopyridine, pyridine, nicotine and sulphapyridine.

The following miscellaneous compounds also gave no fluorimeter readings when tested in biological concentrations: adenosine, adenylic acid, adenosinetriphosphoric acid (ATP), nucleic acid, indole-3-acetic acid, 1-methyl indole acetic acid, glucose, urea, uric acid, *p*-aminobenzoic acid, inositol, pyridoxine, aneurin, riboflavin, pantothenic acid and choline.

Schweigert (1947) has reported that indole interferes in the acetone condensation method of Huff *et al.* (1945), itself giving rise to a fluorescence. We have obtained a similar fluorescence when testing indole with the acetone condensation method (Table 7), but the fluorescence was also present in the 'no NaOH blank', and could thus be eliminated. However, when using our methyl ethyl ketone method little or no fluorescence was given by indole when tested at a concentration of 5 μg./ml. final dilution. When using the 'no NaOH blank' which we recommend in our procedure, indole does not interfere with the estimation of *N*-MN.

Folic acid, pyridoxal and the oxidation product of pyridoxine at neutral pH gave a definite blue fluorescence. This, however, remained in all the blanks and so did not interfere with the *N*-MN results.

Differentiation of N-*MN from coenzyme* I *and* N^1-*methylnicotinuric acid betaine*

Diphosphopyridine nucleotide preparations when tested gave a fluorescence. The fluorescent material, however, unlike *N*-MN, was not adsorbed on to the decalso column in the techniques described above (Table 8). Similarly, N^1-methylnicotinuric acid betaine gave a fluorescence, but was not adsorbed on the column (Table 9). This technique (Procedure 2) allowed a separate estimation of *N*-MN and

Table 7. *Specificity test with indole*

(*a*) Acetone-condensation method

	Fluorimeter readings (mm.)		
Blanks	Indole (5 μg./ml.)	Indole (5 μg./ml.) + *N*-MN (0·1 μg./ml.)	*N*-MN (0·1 μg./ml.)
(1) Water	1	1	1
(2) No NaOH	12	12	1
Full fluorescence	13·5	31	26·5
Blanks	(*b*) Methyl ethyl ketone method		
(1) Water	2	1	1
(2) No NaOH	3	2	1
Full fluorescence	3·5	45	44

Table 8. *Differentiation of* N-*MN from coenzyme* I (Co I) *by decalso filtration*

	Fluorimeter reading (mm.)		
Material	Procedure 1*	Procedure 2†	Difference due to Co I
N-MN, 0·1 μg./ml.	30·0	30·0	0
Co I (50% purity), 1 μg./ml.	26·5	−0·7	27·2
N-MN, 0·1 μg./ml. + Co I (50% purity), 1 μg./ml.	55·2	29·0	26·2

* 'No NaOH blank' procedure measures total fluorescence.
† 'Decalso blank' procedure measures *N*-MN fluorescence.

Table 9. *Fluorescence of* N^1-*methylnicotinuric acid betaine with and without decalso treatment*

	Fluorimeter readings (mm.)			
Material	No NaOH blank	Full fluorescence	Increase	Percentage adsorbed on decalso
N-MN (0·25 μg./ml.)	1·5	105·5	104	—
N-MN, decalso filtered (0·25 μg./ml.)	1·5	1·5	0	100
N^1-methylnicotinuric acid betaine (0·31 μg./ml.)*	2	26	24	—
N^1-methylnicotinuric acid betaine, decalso filtered (0·31 μg./ml.)	2	26	24	0

* Equimolecular to 0·25 μg. of *N*-MN. Prepared by Dr F. Bergel; m.p. 240°. (Found: C, 51, 22; H, 5·57; N, 13·4%. Calc.: C, 50·9; H, 5·66; N, 13·2%.)

the other N^1-substitute pyridine compounds. None of these substances gave a fluorescence with the 'no NaOH blank'. Hence, Procedure 1 (using 'no NaOH blank') gives a measure of the total fluorescence, while Procedure 2 (using 'decalso blank') measures only the fluorescence of *N*-MN. The difference between the two values is therefore a measure of the Co I or N^1-methylnicotinuric acid betaine content. The latter substance has so far as we know not been reported to occur in nature.

In studying a number of preparations of Co I of varying purity, we have found that the fluorescence given by the samples was not strictly related to its coenzyme content as determined spectrophotometrically or enzymically. It is possible that inactive breakdown products may give rise to fluorescence. This matter is under investigation and will be reported elsewhere.

RESULTS

The method has now been used for the determination of *N*-MN in several hundred samples of rat urines, and also in several examinations of human urines before and after test dosing with nicotinamide. Attempts were also made to estimate *N*-MN in rat livers.

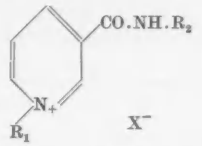
Table 10. *N-MN content of urines and livers. Results obtained with procedures* 1 *and* 2

	Procedure 1 *N*-MN (mg./day)	Procedure 2 *N*-MN (mg./day)
Urines (24 hr.) from a hospital patient:		
1st day	4·8	4·5
2nd day	3·8	3·1
After daily dosing with 100 mg. nicotinic acid:		
1st day	21·3	20·6
2nd day	37·4	36·8
Urines (24 hr.)/rat on the following diets:		
(*a*) 20% casein diet	0·29	0·29
(*b*) 7% casein, 40% maize diet	0·004	0·004
(*c*) 7% casein, 40% maize diet + 0·3 mg. nicotinic acid/day	0·043	0·043
	N-MN (mg./100 g.)	*N*-MN (mg./100 g.)
Specimens of fresh rat liver:		
(*a*) Stock rat 1	6·2	1·6
(*b*) Stock rat 2	6·2	0·8

Table 10 shows that the use of the different blanks gave similar final values in the case of urine, and that the small differences remained fairly constant over a wide range of *N*-MN excretion. In the case of liver, however, Procedure 2 ('decalso blank') showed a far smaller value than the other procedure. Most of the fluorescence therefore was produced by a material which was not adsorbed on the decalso column. This compound, or compounds, showed the properties of Co I rather than *N*-MN.

DISCUSSION

The method as given above produces fluorescent substances probably from all compounds of the type



when treated with alkali and methyl ethyl ketone. Thus Co I and N^1-methylnicotinuric acid betaine give a distinct fluorescence.

The validity of the method depends upon the choice of a suitable blank procedure. Several possibilities have been examined. The procedure using the 'water blank' estimates materials which fluoresce on treatment with ketone but not without ketone; the procedure using the 'no NaOH blank' measures materials which fluoresce in ketone at acid pH only after a period in alkali; the procedure using the 'decalso blank' measures materials fluorescing with ketone under the conditions of the test, and which are also adsorbed on decalso.

It was found the Co I and N^1-methylnicotinuric acid betaine behave like *N*-MN with the 'water blank' and 'no NaOH blank' giving thus an apparent reading of *N*-MN. They were however not adsorbed on decalso and therefore it is possible to differentiate these two compounds from *N*-MN. For routine estimation of material where significant interference by coenzymes is not encountered the simpler procedure using the 'no NaOH blank' may be used.

In rat urine, Procedures 1 and 2 give identical results within the limits of experimental error. In human urine there was a fairly constant difference which did not increase on dosing with nicotinamide and may be explained by a small concentration of coenzyme in the urine. The difference of fluorescence between the 'water blank' and 'no NaOH blank' is also fairly constant and unaffected by dosing with nicotinamide. Thus it is apparently not connected with nicotinic acid metabolism.

The excretion of *N*-MN by rats maintained on various diets, similar to those reported by Kodicek, Carpenter & Harris (1946, 1947), has been examined by this method. The ingestion of maize was found to lower the *N*-MN excretion to one-twentieth of that of rats on a simple casein-sucrose diet of similar protein and nicotinic acid content. Addition of nicotinic acid to the maize-containing diets increased the excretion tenfold. Similar findings, though without changes of such magnitude, have been reported by Ellinger (1946) and other workers.

SUMMARY

1. A rapid and simple method for the estimation of N^1-methylnicotinamide is presented. It has been modified from the procedure described by Huff *et al.* (1945) by using methyl ethyl ketone instead of acetone, and by the adoption of two alternative blank procedures.

2. The conditions of the reaction and the specificity of the method have been studied.

3. With the blank procedures described the fluorescence due to coenzyme I and to N^1-methylnicotinuric acid betaine could be differentiated from that of N^1-methylnicotinamide.

4. Results of N^1-methylnicotinamide estimations have been reported for rat and human urines.

Our thanks are due to Dr Dorothy Needham and Dr W. K. Slater for kindly supplying us with samples of Co I. We are grateful to Dr F. Bergel of Roche Products, Ltd. for his synthesis of N^1-methylnicotinuric acid betaine.

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Studies in Immunochemistry

8. THE ISOLATION AND PROPERTIES OF THE HUMAN BLOOD-GROUP A SUBSTANCE

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The earliest attempts to isolate and identify the substances responsible for the human specific blood-group characters were made by extracting erythrocytes with ethanol (Brahn & Schiff, 1926; Dold & Rosenberg, 1928; Hallauer, 1929, 1932, 1934; Ottensooser, 1932; Stepanov, Kuzin, Markageva & Kosyakov, 1940). The group materials obtained were inhomogeneous, but the results demonstrated that the group substances could be isolated from red cells and were most probably of carbohydrate nature. There are special difficulties associated with the isolation of group substances from erythrocytes and it is now known that these materials are in some way bound up with the lipid constituents of the red-cell surface and are thus rendered water insoluble. Furthermore, the amount of the group substance present in the erythrocyte stroma is exceedingly small. For these and other reasons, methods for the isolation of the specific substances from human red cells are largely undeveloped, and little is known concerning the exact chemical nature of the group-specific complexes on the erythrocyte surface.

The occurrence of the human blood-group substances in a water-soluble form in the tissue fluids and secretions of some individuals has been known for many years and our present knowledge of the human group substances has been obtained largely through a study of these water-soluble components. Landsteiner & Harte (1941) isolated from saliva substances of mucoid nature which possessed group-specific serological characters, and similar materials were obtained from human urine by Freudenberg & Eichel (1934, 1935) and by Freudenberg & Molter (1939). Witebsky & Klendshoj (1940, 1941) likewise isolated carbohydrate materials from the gastric juice of persons belonging to groups **B** and **O**, but they were unable to obtain the group substances free from contaminating materials owing to the small amount of the specific factors available and the absence, at that time,

of methods suitable for the separation of mucoids from proteins and other tissue constituents occurring in the secretions.

A careful study by Meyer, Smyth & Palmer (1937) of the glycoproteins present in pig gastric mucin revealed the presence of a neutral mucopolysaccharide which possessed an intense serological activity corresponding in specificity to that shown by the human group **A** substance, and a similar material was subsequently isolated from commercial peptone by Goebel (1938). Freudenberg & Westphal (1938) also obtained from this source a polysaccharide which possessed group **A** character, and it is now known that the tissues and secretions of certain animals, more especially of pig, horse and cattle, contain substances which carry serological specificities very similar to, or identical with, those shown by the human group substances.

Meyer *et al.* (1937) and Landsteiner & Harte (1940) considered that the neutral polysaccharide obtained from pig gastric mucin which possessed group **A** specificity was composed of equimolecular quantities of *N*-acetylglucosamine and galactose. The material gave a positive Ehrlich diazo reaction. As it was not possible to remove completely the components giving this reaction, Meyer *et al.* (1937) believed that, in the original complex, the glucosamine was firmly bound to a polypeptide chain. Other workers reported the presence of amino-acids in their most active preparations, but were uncertain whether these should be considered as true components of the group-specific material. Landsteiner & Harte (1940) recorded that the purified **A** substance isolated from pig gastric mucin contained rather more than a third of its total N in the form of amino-acids. Subsequently, Morgan & King (1943) obtained preparations of **A** substance from pig mucin by entirely different methods and established that a serologically active substance is obtained which contains 35–40% of its N as α-amino-acid N and that the substance is indeed a polysaccharide-amino-acid complex. It is now known that none of these earlier preparations was homogeneous and that the **A** substance isolated from commercial preparations of pig gastric mucin, peptone or pepsin is contaminated with at least one other mucoid. Examination by Bendich, Kabat & Bezer (1946) of the mucoid materials obtained from individual hog-stomach linings demonstrated that, whereas some materials showed intense **A** activity, others lacked this serological character and were in consequence designated as 'inactive'. Aminoff, Morgan & Watkins (1946) obtained similar results, but identified the 'inactive' mucoid as the so-called **O** substance, i.e. a material found in the secretions and body fluids of the majority of secretors, irrespective of their **ABO** group, but in greatest quantity in group **O** secretors (Witebsky & Klendshoj, 1941; Morgan & van Heyningen, 1944; Morgan & Waddell, 1945). This mucoid was designated **H** substance by Morgan & Watkins (1948) to indicate its ubiquitous nature and to differentiate it as a heterogenetic substance which is not a product of the **O** gene.

The investigations of Putkonen (1930) and Hartmann (1941) showed that, of a large number of human fluids and secretions examined, gastric juice and saliva were the most potent source of the group-specific substances. More recently, Kabat, Bendich, Bezer & Beiser (1947) have shown the latter secretion to contain less than 100 mg. of the group substance per litre. The small yield of the group material from these sources prompted Morgan & van Heyningen (1944) to examine pseudomucinous ovarian cyst fluids for their content of blood-group factors and it was found that these pathological growths are frequently a potent source of the specific substances. The volume of individual cyst fluids varies from a few hundred millilitres to several litres, and cysts are sometimes found to contain several grams of the group-active mucoid which, furthermore, is occasionally present in a relatively pure state. A preliminary account of the isolation of human blood-group **A** substance from this source has been given (King & Morgan, 1944) and the immunological properties of the partially purified group material have also been described (Morgan & Watkins, 1944, 1945, 1948; Rainsford & Morgan, 1946).

The present paper describes in detail the isolation and properties of the human blood-group **A** substance obtained from pseudomucinous ovarian cyst fluids and establishes the material, on the basis of physical, chemical and immunological measurements, as being an essentially homogeneous mucoid or polysaccharide-amino-acid complex.

EXPERIMENTAL

A number of methods for the isolation of the group **A** substance have been employed and modified as additional experience demanded. The very variable quantity of the specific substance present and the unknown nature and amount of non-specific materials in the native cyst fluids, necessitates changing the details of the technique of isolation to suit the individual character of the cyst fluid investigated.

Materials and methods

General. The pseudomucinous ovarian cyst fluid, together with a specimen of the patient's blood and saliva, was collected from the hospital as soon as possible after removal of the cyst. The fluid was preserved at 0° in the presence of toluene or dried *in vacuo* from the frozen state. The blood group of the patient was established as A_1 or A_2 according to the reactions of the cells with human or animal *alpha*$_1$ (α_1) agglutinin. The saliva specimen was diluted with an equal volume of saline and boiled for 10 min. immediately after collection. Bacterial contamination was prevented either by the addition of toluene or by keeping the specimen at $-10°$. The concentration of the **A** substance in the cyst fluid and in the saliva was determined by means of the iso-agglutination inhibition test. The patient's erythrocytes, cyst fluid and saliva were also examined for the so-called **O** character (**H** specificity) in similar agglutination or inhibition tests (Morgan & Waddell, 1945; Morgan & Watkins, 1948). About 130 cyst fluids and saliva specimens have been examined and in no instance has the **A** substance been present in the cyst fluid when it was absent from the saliva.

Determination of serological activity. The iso-agglutination and sheep-cell haemolysis tests were carried out as described by Morgan & King (1943). A 'standard' preparation of **A** substance was included in all tests.

Total nitrogen. This was determined by the Kjeldahl method using the apparatus of Markham (1942) and a mixed bromocresol green and methyl red indicator (Ma & Zuazaga, 1942). The N content of the purest preparations was also determined by the Dumas micromethod (Pregl, see Grant, 1945).

Acetyl content. This was determined by the method of Kuhn & Roth (1933) as described by Grant (1945). The percentage recovery of acetyl from *N*-acetylglucosamine after hydrolysis with *p*-toluenesulphonic acid at 100° for increasing periods of hydrolysis is given in Fig. 1, which indicates that the release of acetyl groups is not complete until hydrolysis has proceeded for at least 4 hr., and the results of similar distillation experiments indicated that a heating period of 5 hr. was necessary to obtain the maximum acetyl value for the A substance.

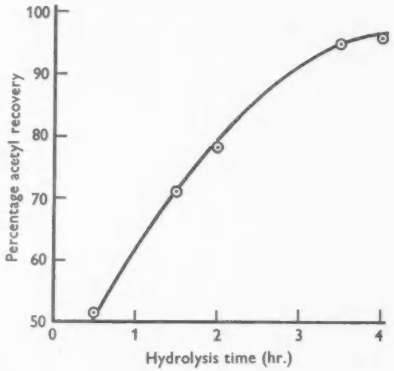


Fig. 1. The liberation of acetyl groups from *N*-acetylglucosamine. The times refer to the times of hydrolysis under reflux; during the subsequent distillation the products are heated for an additional 20 min.

Estimation of the carbohydrate components. *N*-acetylhexosamine was assayed by the method of Morgan & Elson (1934) employing a photoelectric colorimeter. Acetylglucosamine concentrations between 10 and 40 μg./ml. were used as standards. Amino sugar was determined by a modification of the method of Elson & Morgan (1933) using glucosamine concentrations of 10–40 μg. as standards, a photoelectric colorimeter and a green light filter (λ, 540 mμ.). Reducing power was determined as glucose by the copper-reduction method of Somogyi (1937).

The fucose content of the A substance was determined by oxidation of a partial acid hydrolysis product with periodate in Conway units. The method is based on the technique elaborated by Winnick (1942) for the determination of threonine. Thus $NaHSO_3$ (1·5 ml. of a 0·25 M-solution) was pipetted into the central chamber of a Conway unit. The material to be analysed, contained in 0·5 ml. of water, was placed in the outer chamber and 1 ml. of 0·1 M-K_3PO_4 added. The concentration of K_3PO_4 was adjusted so that 1 ml. exactly neutralized, to pH 7·0, the 0·5 ml. of 0·2 M-HIO_4 which was then added. The unit was closed, rotated carefully to mix the solutions in the outer chamber, and allowed to stand at room temperature for 5 hr. A drop of 1% starch was then added to the inner chamber and the excess bisulphite oxidized with I_2 to a faint blue tint. The aldehyde-bound bisulphite was liberated by the addition of powdered Na_2HPO_4 or $NaHCO_3$ and titrated with 0·005 N-I_2. As controls, known amounts of fucose were oxidized in Conway units in a similar manner and the acetaldehyde liberated determined. About 90% of the theoretical amount of acetaldehyde was recovered in the controls.

Estimation of amino-acids. α-Amino-N was determined by measuring the N liberated on treatment with HNO_2 in the Van Slyke-Neill manometric apparatus (Peters & Van Slyke, 1932). α-Amino-acid-N was determined by the ninhydrin method of Van Slyke, Dillon, MacFadyen & Hamilton (1941) at pH 2·5 and a reaction time of 8 min.

Chromatographic methods. Paper chromatography was used for the qualitative examination of the amino-acids, hexosamines and sugars and was employed as described by Consden, Gordon & Martin (1944) using as solvents collidine, phenol and *n*-butanol and Whatman no. 1 (57 × 46·5 cm.) paper. The amino-acids were detected by spraying the papers with ninhydrin. The sugar spots were developed by spraying with $AgNO_3/NH_3$ and heating to 105° for 5 min. (Partridge, 1946). The hexosamines were detected on the paper sheets as cherry red areas, by spraying with a modified alkaline acetylacetone reagent followed by *p*-dimethylaminobenzaldehyde in HCl-*n*-butanol (Partridge, 1948).

The isolation of group A *substance*

Several methods involving the fractionation of the crude cyst material were thoroughly examined. The procedures employed included: (1) Coagulation by heat and removal of the accompanying protein. (2) Fractional precipitation by addition of acetone or ethanol from a solution of the dry cyst material in (*a*) water, (*b*) ethylene glycol, (*c*) diethyleneglycol, (*d*) glycerol and (*e*) formamide. (3) Extraction of the dry cyst material with 90% phenol and subsequent fractionation of the phenol-soluble material by precipitation with increasing amounts of ethanol. (4) Fractional precipitation of an aqueous solution of material insoluble in 90% phenol by sodium sulphate. (5) The removal of free protein by means of trichloroacetic acid. None of these methods alone gives rise to a homogeneous material, but by employing a number of these procedures in a carefully selected order a technique was evolved which gave a material that was essentially homogeneous.

The procedure finally employed for the isolation of A substance, as applied to the cyst fluid from patient no. 84 (group A_1) was as follows: 2 l. of the fluid, which contained about 250 g. of dry substance ($[\alpha]_{5461}$ − 55°; N, 12·9%) were cooled to 0° and, under conditions of vigorous stirring, mixed with an equal volume of 10% trichloroacetic acid. After the material had stood for a short while in a cold room at − 10° it was centrifuged at 0° and the precipitate washed at this temperature with about half the original volume of 10% (w/v) trichloroacetic acid and again collected by centrifugation at 0°. The opalescent supernatant fluid and washings were combined, the solution was centrifuged to remove a small amount of insoluble matter and immediately neutralized at 0° with 10 N-NaOH, care being taken not to exceed pH 7·5. The solution was made slightly acid with acetic acid and vigorously stirred during the addition of an equal volume of ethanol at − 10°. The opalescent solution was then poured into a further two volumes of ethanol at this temperature. The flocculent precipitate that formed was collected after standing at − 10° for

some hours, dissolved in water, dialysed at 0° and dried from the frozen state. Treatment with trichloroacetic acid removed most of the free protein which accompanied the A substance in the native cyst fluid and enabled the subsequent steps in the isolation procedure to be carried out more effectively.

The dry material (10·1 g. $[\alpha]_{5461}+10°$; N, 7·9%) was suspended in 90% (w/v) phenol (200 ml.) and shaken mechanically at intervals during a period of several hours. The material was then centrifuged and the remaining solid extracted with half the original volume of 90% phenol. A third extraction of the residue was necessary to remove the A substance completely. The insoluble material showed little specific serological activity and was rejected. It was observed, however, that with cyst fluids which showed definite H activity (the so-called O character) the material insoluble or very sparingly soluble in phenol contained an enhanced concentration of this mucoid. The combined phenol extracts were then vigorously stirred and fractionally precipitated with ethanol, added in the form of a 1:1 (v/v) 90% liquid phenol-ethanol mixture to avoid local precipitation of the material first thrown out of solution. The colourless, gelatinous fractions which separated from the phenol solution were collected between the ethanol (v/v) levels 0 and 4·5, 4·5 and 8·5, 8·5 and 11, 11 and 17, 17 and 25, 25 and 75%. The largest and most active fraction separated between, 4·5 and 8·5%, weighed 4·07 g. and showed a dextrorotation, $[\alpha]_{5461}+8\pm2°$, and contained 6·3% N. This material was again dissolved in 90% phenol and the fractionation process repeated at the same levels of ethanol concentration. The major part of the material (N, 5·5%) precipitated again between 4·5 and 9·5% ethanol. The substance inhibited iso-agglutination under the test conditions in amounts of the order of 0·4 μg. The material (2·5 g.) was again dissolved in 90% phenol and fractionated from solution by the addition of ethanol. A small fraction (0·022 g. $[\alpha]_{5461}+19°$, N, 4·7%) remained insoluble. The bulk of the material precipitated between the levels 4·5 and 7·5% ethanol and weighed 2·2 g. At this stage in the purification process no further material separated on increasing the ethanol concentration a few per cent, but over the range of ethanol concentration 10–75% (v/v) a small amount (0·09 g.) of material precipitated. This small fraction showed a laevorotation, $[\alpha]_{5461}-3\pm2°$, contained 8·7% N and showed a serological activity equivalent to about 2% of the most active material.

A portion of the main fraction (1·73 g.) was dissolved in 34 ml. of cold anhydrous ethylene glycol and precipitated from solution by the cautious addition of ethanol. No precipitate appeared until a concentration of 42% (v/v) ethanol was reached, and between this level and 47% ethanol the first fraction (0·3 g. N, 6·0%) was collected. Other fractions were removed over the ranges of ethanol concentration 47–50% (0·14 g. $[\alpha]_{5461}+16°$, N, 5·5%); 50–62% (0·60 g. $[\alpha]_{5461}+14°$, N, 5·4%), and 62–75% (0·07 g. $[\alpha]_{5461}+14°$, N, 5·0%). The material remaining in solution (20 mg.) contained 1·4% N.

Another specimen of A substance (3·2 g.) obtained from the same cyst fluid and isolated by means of trichloroacetic acid, phenol and ethylene glycol fractionation as described above was dissolved in neutral anhydrous formamide (50 ml.) and divided into three fractions by precipitation with ethanol. Very little material separated up to 50% (v/v) ethanol concentration. Thereafter material was collected between the ethanol levels 50 and 60% (5·7% N), 60 and 66% (5·8% N) and 66 and 75% (5·8% N). The first fraction contained the major part of the starting material and showed the highest serological activity in the iso-agglutination inhibition test.

To obtain a useful amount of the A substance for detailed examination, materials obtained by the above procedures which showed a N content of about 5·7%, an optical rotation, $[\alpha]_{5461}+15\pm5°$ and a maximum specific serological activity, were pooled and fractionated from aqueous solution by ethanol. The A substance separated almost entirely as a single main fraction within quite narrow limits of ethanol concentration. The materials obtained were dialysed, centrifuged to remove traces of insoluble matter and dried from the frozen state. The analytical figures for the materials which separated during the fractionation of 3·2 g. of A substance are given in Table 1.

Table 1. *The fractionation of* A *substance from aqueous solution by ethanol*

Precipitation levels (% (v/v) ethanol)	Yield (g.)	$[\alpha]_{5461}$	N content (%)
0→52·5	0·02	†	6·08
52·5→57·5	2·90	$+15\pm2°$	5·76
57·5→excess*	0·14	$+9\pm1°$	5·74

* About 4 vol.

† Too opalescent for accurate polarimetric observation.

A preparation of A substance, which was composed of material obtained by the above procedures from several different cysts, was fractionated by the addition of glacial acetic acid to a concentrated aqueous solution of the material and gave a main fraction which separated between the acetic acid levels 92–94% (v/v). The material contained 5·8% N and showed full capacity to inhibit iso-agglutination and the haemolytic activity of rabbit anti-A serum, but was nevertheless found to be somewhat inhomogeneous when examined in the Tiselius electrophoresis apparatus. This preparation, no. III, was used exclusively for the preliminary analytical determinations, the final values given being derived from the best material.

Tests for homogeneity

The following examinations were carried out to establish the homogeneity of the main preparation.

(1) *Fractional solubility test.* Attempts to apply the classical equilibrium method of solubility test failed owing to the ready and extreme solubility of the A substance in water which resulted in a solution of high concentration and considerable viscosity being obtained. For this reason a modified technique was used in which a known weight of A substance was shaken vigorously for a few minutes with successive small amounts of aqueous 30% (v/v) ethanol until all the material had dissolved. After each addition of aqueous ethanol and subsequent shaking, the suspension was centrifuged, the supernatant fluid completely removed and the material in solution recovered by drying *in vacuo* from the frozen state. The fractions so obtained were dissolved to give solutions of known concentration and analysed for total N and for optical rotation. A sample of each fraction was then hydrolysed by heating with

Table 2. *Fractional solubility test for homogeneity: pure* **A** *substance*

Fraction	I	II	III	IV	V	VI
$[\alpha]_{5461}$	+14±6°	—	—	+15±2°	+13±2°	+12±5°
Percentage N (Kjeldahl), *a*	5·67	5·52	5·61	5·58	5·71	5·71
Reduction (% calculated as glucose), *b*	53·7	53·3	53·0	52·6	54·0	53·7
Hexosamine (% calculated as base), *c*	36·7	36·7	37·4	37·4	36·7	37·5
b/*a*	9·48	9·65	9·44	9·44	9·45	9·41
c/*a*	6·47	6·64	6·66	6·71	6·43	6·56
b/*c*	1·46	1·45	1·42	1·41	1·47	1·43

Table 3. *Fractional solubility test for homogeneity: partially purified preparation of* **A** *substance from hog gastric mucin*

Fraction	I	II	III	IV	V
Percentage N (Kjeldahl), *a*	6·05	6·51	6·43	6·54	5·48
Reduction (% calculated as glucose), *b*	51·2	46·8	45·8	46·2	24·2
Hexosamine (% calculated as base), *c*	30·7	32·5	30·2	30·7	19·4
b/*a*	8·46	7·18	7·12	7·06	4·41
c/*a*	5·07	4·99	4·70	4·70	3·54
b/*c*	1·67	1·44	1·52	1·50	1·25

0·5N-hydrochloric acid at 100° for 16 hr. and the reducing power and hexosamine content determined. The values for the reducing power are not the maximum values owing to some decomposition after 16 hr. hydrolysis (Fig. 4). The results (Table 2) show that the analytical figures for each of the six fractions were very similar and that there is no evidence that material differing in composition from the **A** substance is present in the preparation. The results of the application of this technique to a preparation of **A** substance obtained from commercial hog mucin and known to be heterogeneous are set out in Table 3 and reveal at once the different composition of the fractions obtained.

(2) *Electrophoresis.* An electrophoretic analysis of the **A** substance was carried out at pH 4 and 8 and gave no evidence of inhomogeneity. Details are given by Dr R. A. Kekwick in an addendum to this paper.

(3) *Ultracentrifugal examination.* The **A** substance was also examined by Dr Kekwick using the Svedberg oil-turbine centrifuge and the 'diagonal schlieren' optical system. Full experimental details are given in the addendum.

(4) *Quantitative immunochemical analysis.* Through the kindness of Dr E. A. Kabat the group **A** substance was examined by the quantitative precipitation technique. All the hexosamine and fucose present in the **A** substance was specifically precipitated by human anti-**A** serum and thus indicated the absence of significant amounts of other mucoids which contain these carbohydrate constituents (Kabat, Baer & Knaub, 1949).

Properties of the **A** *substance*

Immunological. The serological activity of the **A** substance was measured by determining the minimum amount necessary to inhibit (*a*) the agglutinating action of a standard anti-**A** agglutinin on $\mathbf{A}_1$ cells, and (*b*) the haemolysis of sheep red cells by a rabbit anti-**A** immune serum in the presence of complement. Under strictly controlled conditions both of these tests are reliable and will detect an increase or decrease in activity of the order of 33 %. The minimum amount of **A** substance which will inhibit completely three to four agglutinating doses of anti-**A** agglutinin under the standard test conditions is of the order 0·2 μg. The haemolysis test is considered to measure the 'Forssman' or heterophile component of the **A** agglutinogen and is accepted as measuring a different, although closely related, serological property from that determined by the isoagglutination inhibition technique. The minimum amount of the material which will inhibit haemolysis under the test conditions is 0·05 μg.

The **A** substance was examined for its capacity to inhibit: (1) anti-**B** agglutinin (human); (2) the so-called 'anti-**O**' agglutinins, as exemplified by (*a*) certain normal cattle (Schiff, 1927; Greenfield, 1928) and human sera (Morgan & Watkins, 1948), (*b*) the sera of goats immunized with *Shigella shigae* (Eisler, 1930, 1931) and (*c*) immune rabbit anti-'**O**' sera (Morgan & Waddell, 1945); (3) anti-**M** and anti-**N** sera; (4) anti-*Rhesus* sera, using anti-**D**, anti-**C**, anti-*c* and anti-**E** sera as test reagents; and (5) the 'Lewis' blood-group reagents, anti-Le^a and anti-Le^b sera (Grubb & Morgan, 1949). Tested at a concentration of 5 mg./ml. the **A** substance contained no detectable amounts of material able to neutralize the agglutinins **B**, **M**, **N** and *Rh*. Weak inhibition of **H** agglutinin (the so-called **O** agglutinin) by the **A** substance was first observed at a concentration of 0·2 mg./ml. Under identical test conditions the **H** mucoid isolated from the pseudomucinous ovarian cyst fluids of women belonging to group **O**, inhibited **H** agglutinin at a concentration of 0·1 μg./ml.

Similarly, the A substance at a dilution of 1 : 10,000 showed some activity against the 'Lewis' anti-sera Le^a and Le^b, but this activity represented less than 1 % of the activity shown by the purified Le^a- and Le^b-substances. The A substance does not precipitate with horse pneumococcus Type XIV anti-serum.

The capacity of the purified A substance to induce the formation of anti-A immune body in the rabbit was investigated. A group of six rabbits was given a series of six intravenous doses each of 0·05 mg. of the A substance dissolved in saline. The injections were made at 3–4-day intervals. The average anti-A agglutinin titre of the normal bleedings was 104. After immunization the average titre was 208. Three of the rabbits were given a further three intravenous doses each of 0·1 mg. of the A substance and were bled 1 week after the last dose. The average anti-A agglutinin titre increased to 429. The remaining three rabbits were given under the same conditions three doses of an artificial antigen (0·05 mg.) made from the same specimen of A substance and the conjugated protein component of the O antigen of *Shigella shigae* as described in an earlier communication (Morgan, 1943). The average anti-A agglutinin titre increased to 5400, a 50-fold increase over the average normal titre and rather more than ten times the value of the average titre after nine doses of the A substance alone. The results indicate that under the conditions of test the A substance possesses weak antigenicity only, but that it can be converted to a powerful antigen by combination with the conjugated protein component of the O antigen of *S. shigae*.

Physical. The relative viscosity, η, of a 0·5 % solution of the A substance in 0·85 % saline is 1·14. An elastic gel is not formed on the addition of borate buffer, pH 8·5, to a 1 % solution of the A substance. The specific rotation, the mean value obtained from the examination of several preparations, is $[\alpha]_{5461} + 15 \pm 5°$ in water (c, 1·0).

The ultraviolet absorption of the A substance was examined in a Beckmann Model D.U. quartz spectrophotometer. The concentration of the solution was 0·1 % in 0·85 % NaCl and the observations were made in a cell 1 cm. deep. The absorption curve is given in Fig. 2. There is no evidence of absorption between the wavelengths 220 and 260 mμ. It is doubtful if the very weak absorption between 260 and 300 mμ. is significant.

Chemical. An aqueous (2 %) solution of the purified A substance gives no precipitate when treated with an equal volume of 2 % solutions of the following reagents: aluminium sulphate, potassium aluminium alum, lead acetate in dilute acetic acid or alkali, mercuric acetate, uranium acetate, silver nitrate, mercuric sulphate in 5N-H_2SO_4, phosphotungstic acid, neutral and acid copper sulphate, hydroferrocyanic acid, picric acid, trichloroacetic acid (10 %), tannic acid (10 %) and salicylsulphonic acid (20 %). The addition of an equal volume of a saturated solution of magnesium or ammonium sulphate gives no precipitate, but these reagents at full saturation give rise to some turbidity in the solution on warming. An aqueous solution of the A substance alone gives no precipitate on boiling. The biuret, ninhydrin, xanthoproteic, Millon, Hopkins-Cole, Selivanoff and Bial tests are negative. Ehrlich's diazosulphanilic acid test is faintly positive as is Sakaguchi's tests for arginine. The addition of alkaline copper sulphate to a solution of the A substance, as in the biuret test, gives rise to a voluminous precipitate.

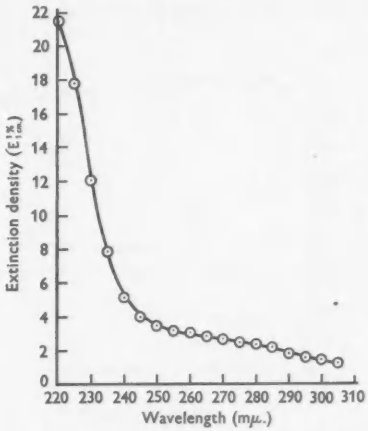


Fig. 2. The ultraviolet absorption spectrum of A substance (0·1 % solution in 0·85 % NaCl).

The analytical figures for a specimen of A substance are: C, 44·2; H, 6·96; N, 5·72 % (Dumas). The material contains less than 0·05 % P and S. The ash content varies somewhat for different preparations, but is usually less than 1 %. The acetyl content falls within the range 8·8–9·1 % for different preparations.

Under the conditions for the determination of *N*-acetylglucosamine (Morgan & Elson, 1934) the A substance readily gives a colour with Ehrlich's reagent. The maximum colour given by *N*-acetylglucosamine, however, is obtained after 4 min. heating at 100° with the 0·05N-sodium carbonate, whereas the A substance requires 14 min. heating under the same conditions and the colour obtained is equivalent to 10·4 % of *N*-acetylglucosamine. Further heating with the sodium carbonate results in a decreased colour production as a consequence of the decomposition of the very labile chromophoric group. Correcting for this decomposition, by extrapolation to zero time, the colour given by the A substance is equivalent to an *N*-acetylglucosamine content of 13·5 %.

Acid hydrolysis of the **A** *substance*

The **A** substance was hydrolysed with 0·5 N- and 6N-hydrochloric acid, and N-acetic acid. The hydrolyses were carried out in small (2–3 ml.) sealed glass ampoules which were heated for the appropriate length of time in a boiling water bath. The ampoules were then cooled and the contents evaporated to dryness *in vacuo* in small glass dishes over pellets of sodium hydroxide and conc. sulphuric acid. Small amounts of sodium chloride do not interfere with the hexosamine or reduction determinations and the 0·5N-hydrochloric acid hydrolysates were therefore not evaporated to dryness but neutralized with an equal volume of 0·5N-sodium hydroxide.

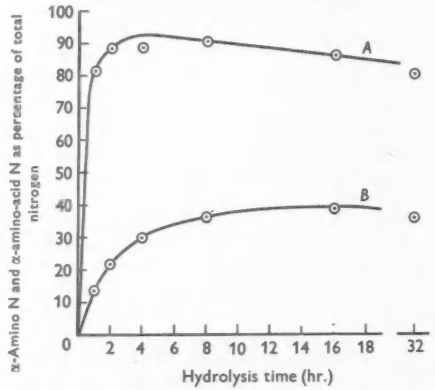


Fig. 3. Hydrolysis of **A** substance by 6N-HCl at 100° as determined by the liberation of (*A*) α-amino groups, and (*B*) α-amino-acids.

Hydrolysis with 6N-hydrochloric acid gave rise to extensive decomposition with the formation of a precipitate of humin particles after 2 hr. heating. The course of the hydrolysis was followed by measuring the α-amino-N and the α-amino acid-N liberated. The results (Fig. 3) show that the liberation of α-amino-N reaches its maximum value in 4 hr. and represents about 91 % of the total N of the substance, whereas the α-amino acid-N reaches its maximum after 16 hr. heating and is equivalent to about 38 % of the total N. The difference between the analytical values for the two forms of nitrogen is due to the hexosamines which react quantitatively with nitrous acid, but do not liberate carbon dioxide on heating with ninhydrin.

The hydrolysis with 0·5N-hydrochloric acid results in the ready liberation of reducing substances and hexosamines. The analytical results (Fig. 4) indicate that the maximum liberation of hexosamines, 37 % (as base), and of reducing sugars, 57 % (expressed as glucose), occur at approximately the same time (4–5 hr.). Fucose is readily liberated and the maximum value 13·5 %, determined as acetaldehyde after periodate oxidation, is obtained after the first hour of hydrolysis. More prolonged hydrolysis results in some decomposition of the fucose, but the apparent increase in the fucose content in the later stages of hydrolysis could be attributed to the liberation of threonine in a form susceptible to oxidation.

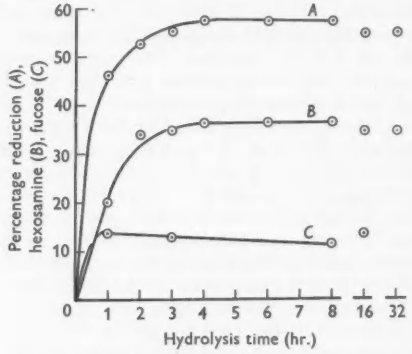


Fig. 4. Hydrolysis of **A** substance by 0·5N-HCl at 100° as determined by (*A*) reducing power, expressed as glucose, (*B*) hexosamine, expressed as glucosamine base, and (*C*) fucose, determined as acetaldehyde after oxidation with periodate.

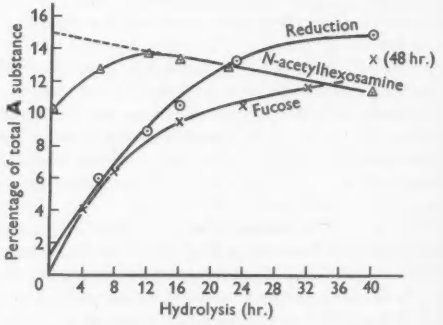


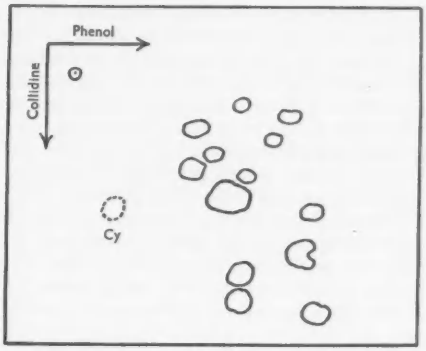
Fig. 5. Hydrolysis of the **A** substance by N-acetic acid at 100° as determined by (*A*) reducing power, expressed as glucose, (*B*) *N*-acetylhexosamine, and (*C*) fucose, determined as acetaldehyde after oxidation with periodate.

Hydrolysis of the **A** substance with N-acetic acid at 100° allowed the rate and extent of liberation of fucose, reducing substances and *N*-acetylhexosamine residues to be determined and the results are set out in Fig. 5. The rate of liberation of fucose indicates that a maximum value 12·9 % is obtained after about 48 hr. hydrolysis and the maximum figure for reducing substances is reached at about the

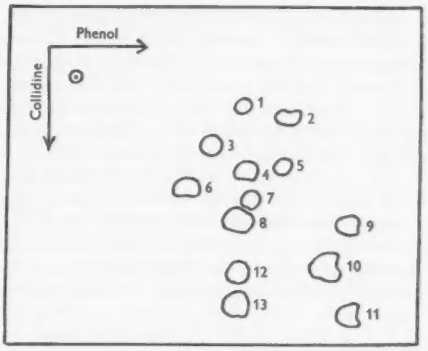
same time. The colour given with Ehrlich reagent, after the hydrolysis products have been treated with dilute sodium carbonate, rises to a maximum value of about 15% expressed in terms of *N*-acetylglucosamine. Simultaneous with these changes there is a steady loss of **A** activity as measured by the ability to inhibit iso-agglutination, but the capacity to inhibit the haemolysis of sheep cells by rabbit anti-A serum is not only retained but enhanced. Moreover, whereas the undegraded **A** substance gives no precipitate with horse anti-pneumococcal Type XIV serum the mild acid hydrolysis products readily yield a precipitate. The results of further examination of the products obtained after simple dialysis of a 16 hr. N-acetic acid hydrolysate have already been reported (Aminoff, Morgan & Watkins, 1948).

The products of complete hydrolysis with 6N-hydrochloric acid (16 hr.) were examined by partition chromatography using 1 mg. of the material. The chromatogram, run in two directions at right angles with collidine and phenol, gave rise to thirteen 'spots' on treatment with ninhydrin reagent containing 1% acetic acid (Consden & Gordon, 1948), and subsequent heating of the chromatogram at 80–90° for 5 min. revealed no further coloured areas, thus indicating the absence of polypeptides in the hydrolysis products. The distribution of the 'spots' on the chromatogram is set out diagrammatically in Fig. 6*A*. Eleven of the 'spots' corresponded closely in position with those which were given by the following amino-acids: Lysine (1), arginine (2), aspartic acid (3), glutamic acid (4), glycine (5), serine (6), alanine (7), threonine (8), proline (9), valine (10), leucine and isoleucine (11) as shown in the chromatogram, Fig. 6*B*. An additional spot corresponding in position and colour with cysteic acid (*Cy*) appeared only occasionally and was then extemely weak. Two spots (12 and 13), not normally found in protein hydrolysis products, appeared on the chromatogram of the hydrolysate of the **A** substance (Fig. 6*A*).

In view of the known reactions of glucosamine and chondrosamine with ninhydrin, both of which give a greyish mauve coloured area on paper after heating at 90° similar to those developed by amino-acids, and the known presence of hexosamine in the **A** substance, evidence for the identity or non-identity of the unknown 'spots' with those that would arise from glucosamine or chondrosamine was sought. The experiments carried out to establish the presence of the two amino sugars have already been described (Aminoff & Morgan, 1948), and the chromatographic evidence that two amino sugars are present may be summarized as follows: (1) two greyish mauve spots, which do not occupy exactly the same position as any of the known amino-acids, develop on treatment with ninhydrin. (2) The same areas (12 and 13) give rise to cherry-red spots on treatment with acetyl-acetone and Ehrlich reagents as described by Partridge (1948), and reduce the silver nitrate-ammonia reagent (Partridge, 1946). (3) The position of the two spots correspond exactly with those which develop on paper when chondrosamine and glucosamine (12 and 13 in Fig. 6*B*) are used as test substances and are treated with solvents under the same conditions; that is, the unknown substances have the same R_F values as glucosamine and chondrosamine.



(*A*)



(*B*)

Fig. 6. Two-dimensional chromatograms of (*A*) the products of the **A** substance obtained by hydrolysis with 6N-HCl for 16 hr. and (*B*) an artificial mixture of amino-acids and hexosamines. Developed in collidine for 48 hr. followed by 24 hr. in phenol/coal gas/ammonia 0·6% (w/v).

Chromatograms run with collidine and phenol have been used to detect the presence of reducing sugars using the silver nitrate-ammonia reagent, but in our experience the traces of phenol which remain in the paper interfere with the reaction. Although collidine gives a useful separation of most of the constituents, the chloride ion has the same R_F value as

galactose in this solvent and there is in consequence a masking of the diffuse brownish spot given by this sugar when the silver nitrate-ammonia reagent is employed. The replacement of collidine with butanol gave rise to chromatograms which after spraying with silver nitrate-ammonia developed three brownish coloured areas, one of which was identified as hexosamine (glucosamine and chondrosamine have almost identical R_F values in this solvent). It was observed, however, that the hexosamine reagents also detected galactose and fucose as faint transient greyish blue spots when butanol was used as solvent. A white precipitate of silver chloride no longer interfered with the development of the brown area due to galactose but the similar mobilities of galactose and hexosamines in butanol now caused these two sugars to be poorly separated from each other. In an atmosphere of acetic acid (3%) the mobilities of the hexosamines are reduced and the galactose is in consequence more easily separated and detected. The distinctive colours given by the hexosamines (pink) and fucose and galactose (greyish blue) on development with the hexosamine reagents serve to distinguish the glucosamine from the galactose when these sugars occur close together on the chromatogram.

Some preliminary observations have been made using partial hydrolysis products of the A substances obtained during the early stages (2, 4 and 8 hr.) of hydrolysis with 6N-hydrochloric acid. The chromatograms indicated that after 2 hr. hydrolysis a small amount of each amino-acid had been liberated. The colour intensity of the spots was much less than that observed with the 16 and 32 hr. hydrolysis products, but nevertheless the chromatograms were qualitatively similar. Heating the chromatograms at 90° for 5 min. caused no further well-defined coloured areas to develop, but induced the formation of a weak and diffuse coloration as a background suggesting the presence of many polypeptide units. The results obtained with the 16 hr. 0·5N-hydrochloric acid hydrolysis products indicated the absence of the leucine/isoleucine spot whilst serine, threonine, proline and valine were present in small quantities only.

The diffusate from the 16 hr. N-acetic acid hydrolysis product of the A substance when run in collidine and sprayed with ninhydrin, silver nitrate-ammonia and the hexosamine reagents, gave five distinct spots of which only that due to fucose persisted in the 0·5N-hydrochloric acid hydrolysate of the material, thus indicating the complex nature of the other components in the diffusate. The results of the chromatographic and quantitative analysis of the diffusible and indiffusible fractions from 16 hr. N-acetic acid hydrolysate after further hydrolysis with 0·5 and 6N-hydrochloric acid can be summarized as follows. The diffusate contains free fucose, and a small amount of both hexosamines and of the amino-acids except the leucines, proline and traces of the hydroxyamino-acids. The indiffusible material contains galactose and about 80% of the total hexosamines, but fucose was not detected. All the amino-acids initially present in the A substance are represented in this material which contains at least 85% of the total amino-acid N in the A substance.

DISCUSSION

The procedure described for the isolation of the specific blood-group A substance from human ovarian cyst fluids has enabled useful amounts of this important tissue component to be obtained and certain of its physical, chemical and immunological properties to be recorded. Owing to the very variable nature of the ovarian cyst fluids an exact description of an isolation procedure which will be completely successful with all fluids cannot be given, but in our experience it has only been difficult to isolate A substance of high specific activity from fluids which contain relatively low concentrations of the group substance and high concentrations of contaminating protein or other mucoid. No attempt has been made to recover group material from paramucinous cyst fluids, the 'trembling jelly' type of cyst contents first described by Mitjukoff (1895).

In some instances the preliminary treatment of the diluted cyst fluid with trichloroacetic acid to remove excess protein is unnecessary, and direct extraction of the freeze-dried cyst fluid with 90% phenol allows the specific material to be readily recovered in a relatively pure condition. It is to be noted, however, that extraction of the freeze-dried native cyst fluid with 90% phenol sometimes yields the A substance in a form in which it is sparingly soluble in this solvent. Treatment of this material with trichloroacetic acid at 0° is then necessary and converts the A substance to a form which is soluble in phenol and which can then be fractionally precipitated by the addition of ethanol in the usual manner. In our experience, if homogeneous material is to be obtained, fractionation of the A substance from phenol solution by ethanol must be carried out within narrower limits of ethanol concentration than 0 and 10% as described in an earlier paper (Morgan & King, 1943) and employed by Bendich *et al.* (1946) and by Kabat *et al.* (1947).

Preparations of A substance which have been repeatedly fractionated from solution in 90% phenol with ethanol and which have remained unchanged in their physical, chemical and serological properties, on further fractionation from solution in anhydrous ethylene glycol or formamide by ethanol have likewise shown no change in composition and specific activity. Material obtained in this way shows only one component when examined in the Tiselius

electrophoresis apparatus at pH 4 and 8. Examination in the Svedberg ultra-centrifuge reveals that the material is polydisperse and that at a concentration of 0·5 % the mean sedimentation constant of the material is S_{20}, $6{\cdot}7 \times 10^{13}$.

Quantitative immunochemical analysis indicates that the material is essentially free from other materials which contain hexosamines or fucose. This conclusion is supported by the absence of the capacity of the A substance to inhibit anti-sera for the other blood-group factors.

The constancy of the physical, chemical and serological data obtained for material purified by the above methods indicates that the A substance described is most probably a homogeneous molecular complex. As such the material is suitable for detailed chemical and immunological study, including step-wise or complete degradation to its constituent residues. If any significance is to be attached to the isolation of certain substances, present in small amounts only, the homogeneity of the material investigated is of prime importance, a fact which is frequently overlooked. The material is, therefore, being examined further by adsorption frontal analysis according to the general procedure described by Claesson (1946) and by the counter-current distribution technique of Craig (1944).

The properties of the A substance reveal that the group A complex is a more stable molecular species than is the chemically similar O antigenic complex of *Shigella shigae* or *Salmonella typhi*, for it is observed that solvent systems which bring about dissociation of these complexes into their component residues fail completely to accomplish any separation of the A complex into its polysaccharide and amino-acid moieties. The A substance must therefore be considered as a complex mucoid which contains a polysaccharide component firmly linked to an amino-acid containing residue.

The undegraded A substance shows two serological properties; the capacity to inhibit the agglutination of group A red cells by human anti-A (α) serum and the power to inhibit the haemolysis of sheep red cells by rabbit immune anti-A serum, and these have been used to determine the amount of the A substance in a given preparation. Since the heterophile activity shows a large increase during the early stages of hydrolysis with N-acetic acid or after treatment with formamide at 150° (Landsteiner & Harte, 1940) the enhancement is most probably due, as suggested by these authors, to a structural change in the complex rather than to the presence of an acid-stable impurity. Contrary to the observation of Jorpes & Norlin (1933, 1934), Jorpes (1934), Jorpes & Thaning (1945) it has not been found possible to fractionate the A substance into two components, each showing one only of the two serological properties associated with the A substance. In one experiment a small fraction of the A substance was soluble in glacial acetic acid and, determined by the two serological methods, the ratio of the activities of the soluble and insoluble fractions was the same as for the original A substance. Furthermore, the ratio of the two serological activities for the most highly purified specimen of A substance, obtained after the application of the various purification procedures described, was the same as for the A substance in the native cyst fluid. Although the inhibition of haemolysis is a more sensitive means of measuring A activity than is inhibition of iso-agglutination, the latter determines a more labile serological character and hence in order to detect any degradation it is advisable to follow the course of purification of the A substance by both methods.

Our preparations contain neither phosphorus nor sulphur in significant amounts and thus differ in this respect from those isolated from pooled hog stomachs by Kabat, Bendich & Bezer (1946) and described by them as containing up to 1·0 % P and 0·4 % S. It is also to be noted that the A substance obtained from human saliva by Kabat *et al.* (1947) likewise differs from our purified material in showing a laevorotation whereas the A substance we have obtained from human ovarian cyst fluids is dextrorotatory, when obtained free from impurities.

The amino-acid-containing moiety of the A complex contains at least eleven amino-acids. The aromatic and sulphur-containing amino-acids are absent or are present in extremely small amounts, whereas threonine occurs in a higher concentration than has been recorded for most proteins. Threonine has been isolated in crystalline form from a purified preparation of human A substance by Freudenberg, Walch & Molter (1942).

Complete hydrolysis of the A substance with mineral acid at 100° liberates reducing sugars and amino-acids. The maximum values found indicate that about 38 % of the total N is present as α-amino-acid N, 51 % as hexosamine N and 91 % as α-amino N.

The polysaccharide component contains D-galactose, L-fucose—first identified as a constituent of the blood-group A substance obtained from pepsin by Bray, Henry & Stacey (1946)—and two hexosamines, D-glucosamine and most probably D-chondrosamine. The recognition that two different hexosamine molecules are present in a molecular species, which is apparently homogeneous, is of considerable interest, and suggests that a re-examination of the amino sugar components of other homogeneous mucopolysaccharides and mucoids is desirable, especially where the recovery of glucosamine is poor in terms of the amount of hexosamine believed to be present on the basis of the colorimetric determination.

The fucose content of the A substance, based on the yield of acetaldehyde liberated on oxidation with

periodate after mild acid hydrolysis is found to be about 13 %. Kabat *et al.* (1949), however, using the colorimetric method of Dische & Shettles (1948) give a considerably higher figure, 18 %, for our preparation of **A** substance. Assuming that this higher figure represents the total fucose content of the **A** substance these results indicate that only a part of the total fucose is in the form of acid-labile end groups.

The hexosamine content of the **A** substance is 37 %, but a slightly higher figure than this is frequently obtained after hydrolysis with 6N-hydrochloric acid. The increased colour (2–3 %) which arises after treatment with strong acid is believed to be due to the formation of chromogenic complexes between sugars or their degradation products and certain amino-acids, e.g. lysine. The hexosamine value, 37 %, expressed as *N*-acetylhexosamine represents 46 % of the **A** substance and thus would account for the total *N*-acetyl groups, 9 %, found to be present. Unhydrolysed **A** substance gives rise to a colour equivalent to about 13 % *N*-acetylglucosamine which represents only a quarter of the total *N*-acetylhexosamine residues calculated to be present, thus indicating that the *N*-acetylhexosamine units are combined by means of at least two different types of linkage.

The galactose content of the **A** substance was not determined, but an approximate value for the amount present can be obtained indirectly by substracting the contribution of the other carbohydrate constituents, chondrosamine, glucosamine and fucose, from the total reducing value of the hydrolysate. The galactose is found to be present to the extent of a maximum of about 17 % and hence it may be considered that, contrary to the observations of Meyer *et al.* (1937) and Landsteiner & Harte (1940), *N*-acetylhexosamine and galactose are not present in equimolecular proportions.

The results of the hydrolysis of the human blood-group **A** substance with 0·5 N- and 6N-hydrochloric acid and subsequent qualitative and quantitative analysis of the hydrolysis products have given an indication of the nature and amount of the constituents present. Additional information as to the stability of the linkages and mode of combination of the components has been obtained from a study of the partial acid hydrolysis products of the material. The results, which have been briefly reported by Aminoff *et al.* (1948) demonstrate that the **A** substance shows no initial reactivity with horse anti-pneumococcal, type XIV, serum, but develops this serological property during the course of hydrolysis with acetic acid. Kabat, Bendich, Bezer & Knaub (1948) and Kabat, Baer, Bezer & Knaub (1948), who independently studied the same problem, state that all their preparations of **A** substance show pneumococcal type XIV specificity. In this important respect, therefore, the **A** substance described by Kabat and his colleagues (1948) differs from our material. These workers also showed that the so-called **O** substance likewise develops increased pneumococcal type XIV specificity as a result of controlled acid hydrolysis (Kabat, Bendich, Bezer & Knaub, 1948) and it would appear that the **A** and **H** (so-called '**O**') substances possess a structure common to both and to the pneumococcus type XIV polysaccharide. The serological differences in the **A** and **H** substances are, therefore, presumably associated with the acid-labile moiety.

The complete interpretation of the results of the action of N-acetic acid on the **A** substance is difficult on account of the complexity of the hydrolysis products. The unhydrolysed diffusate contains at least five components, four of which are still composed of several constituents. Fucose is present as such. The diffusate, which shows only one ninhydrin reacting spot, gives rise to several amino-acids after hydrolysis with 6N-hydrochloric acid. The polypeptide component contains small amounts of the individual amino-acids present in the original **A** substance, with the exception of the leucines and proline, whereas the hydroxyamino-acids, serine and threonine, are present in minute amounts only. It may be assumed, therefore, that these amino-acids are more firmly bound than the others in the **A** substance.

The **A** substance, after hydrolysis with acetic acid, shows an increased capacity to react with Ehrlich reagent under the conditions used for the determination of *N*-acetylglucosamine and 82 % of the *N*-acetylhexosamine units which give this reaction are present in the indiffusible material (Aminoff *et al.* 1948). There is, therefore, an increase in the number of *N*-acetylhexosamine residues present in the reactive form, and these *N*-acetylhexosamine units are attached to the more stable moiety of the **A** substance. Mild alkaline hydrolysis of the **A** substance (Morgan, 1946), on the other hand, renders most of the *N*-acetylhexosamine residues which give immediate colour withEhrlich reagent readily diffusible and therefore alkaline hydrolysis would seem to involve the rupture of different linkages in the **A** substance from those attacked by acetic acid.

In contrast it is found that after treatment with N-acetic acid, the greater part of the fucose in the **A** substance is present in the diffusate as a free sugar. Since most of the fucose is readily liberated without extensive splitting of other glycosidic bonds, it seems reasonable to conclude that these labile fucose units are present as terminal residues and are probably attached to the *N*-acetylhexosamine molecules which are exposed as a result of the mild acid hydrolysis.

The results of the qualitative and quantitative examination of the acetic acid hydrolysis products of **A** substance show that (*a*) about three-quarters of the total fucose is liberated, (*b*) there is a rapid and

complete destruction of the capacity of the A substance to inhibit iso-agglutination, (*c*) there is an increase in its power to prevent the haemolysis of sheep cells by rabbit anti-A serum, and (*d*) a new serological character, that of reacting with horse anti-pneumococcus, type XIV, serum develops. In view of these observations, the possibility that degradation has occurred cannot be readily excluded for preparations of A substance showing any properties that could be associated with the above changes, e.g. a low fucose content and a cross-reactivity with horse anti-pneumococcal type XIV serum.

The analytical data indicate the presence of 46 % *N*-acetylhexosamine, 18 % fucose and a maximum of about 17 % galactose and account for the major part of the total complex. The remainder is made up of amino-acids, of which threonine represents the largest component. On the basis of these preliminary analytical figures, it would appear that the monosaccharide units are present in the following proportions: *N*-acetylhexosamine, 2; galactose, 1; and fucose, 1. The weight of the A substance which contains the minimum number of sugar units is 2×484 or 968 (where 484 is the weight of the A substance which contains one hexosamine unit). This is the weight of the repeating unit. From the ultracentrifugal and diffusion data the molecular weight of the A substance is of the order 260,000, and the molecule therefore contains 280 such units.

SUMMARY

1. Methods for the isolation and purification of the human blood-group A substance from pseudomucinous ovarian cyst fluids are described.

2. The A substance is a dextrorotatory mucoid or polysaccharide-amino-acid complex. The material, which is polydisperse, is essentially homogeneous with respect to chemical, immunochemical and serological properties. The acid hydrolysis products contain 57 % reducing substances, expressed as glucose, and 37 % hexosamine. The α-amino-acid nitrogen represents about 38 %, and the α-amino nitrogen, 91 %, of the total nitrogen (5·7 %).

3. From the analytical results it would appear that the simplest unit of the carbohydrate moiety of the A substance contains one fucose, one galactose and two *N*-acetylhexosamine residues, and that there are about 280 such units in the molecule of A substance.

4. The significance of certain changes in the properties of the A substance which are associated with degradation are discussed.

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ADDENDUM

Physico-Chemical Examination of Blood-Group A Substance

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The A substance was examined in the electrophoresis apparatus, and ultracentrifuge, and in addition the partial specific volume and diffusion constant were determined.

METHODS

Electrophoresis. The material was examined in a Tiselius electrophoresis apparatus at 0°, using the diagonal schlieren optical system and monochromatic light, $\lambda = 546$ mμ., isolated from a mercury arc by a suitable filter. As the main object of this examination was to detect any traces of contaminating high molecular substances, solutions of salt-free material were made up in suitable buffers to give a concentration of 2 g./100 ml., and dialysed against buffer overnight.

Sedimentation. The ultracentrifugal examination was carried out in a Svedberg oil turbine machine at 60,000 rev./min. (270,000 *g*) using an optical system similar to that of the electrophoresis apparatus. The variation of sedimentation constant with concentration was examined over a limited concentration range. Solutions were made up in suitable buffers and sufficient NaCl added to suppress any charge effect on sedimentation.

Diffusion. The diffusion measurements were made by Dr A. G. Ogston using the method of Coulson, Cox, Ogston & Philpot (1948).

The partial specific volume. This was determined pyknometrically at 25°, the substance being dissolved in the buffer used for the majority of the sedimentation constant measurements.

RESULTS

In acetate buffer pH 4, $I = 0{\cdot}1$ and in phosphate buffer pH 8, $I = 0{\cdot}2$, after prolonged electrophoresis only one component was demonstrable. At pH 4 the boundaries were symmetrical, but at pH 8 both the ascending and descending boundaries were asymmetrical. Migration in each instance was anodic, but at pH 4 was so slight as to be barely detectable.

In the ultracentrifuge only one component was present at pH 4 and 8. At pH 8 the sedimentation constant was determined at three concentrations and the data are recorded in Table 1. Owing to the spreading of the boundary at low concentrations, it was not possible to make satisfactory measurements below a concentration of 0·5 g./100 ml. It will be observed that there is a marked dependence of sedimentation constant on concentration.

Table 1. *Sedimentation and diffusion constants of group* A *substance*

(Sedimentation and diffusion constants corrected to water at +20° (Svedberg & Pedersen, 1940).)

Concentration (g./100 ml.)	$S_{20} \times 10^{13}$ (corr.)	$D_{20} \times 10^{7}$ (corr.)
0·42	—	1·70
0·50	$6{\cdot}7_0$	—
0·75	$5{\cdot}9_8$	—
0·81	—	1·60
1·00	$5{\cdot}4_2$	—

In Table 1 are included two values for the diffusion constant kindly measured by Dr A. G. Ogston. The derived diffusion curves showed a departure from the Gaussian distribution.

The sedimentation constant was plotted against concentration, and values interpolated corresponding

to the concentrations employed in the diffusion experiments. From these and the experimentally determined value of the partial specific volume, 0·635, the molecular weight was determined from the expression

$$M = \frac{\mathbf{R}TS}{D(1-V\rho)},$$

where M = molecular weight, $\mathbf{R}$ = gas constant, S = sedimentation constant, D = diffusion constant, T = absolute temperature, V = partial specific volume, ρ = density of solvent, resulting in a mean value of 260,000 (Svedberg & Pedersen, 1940). From the same data the frictional ratio f/f_0 was computed, a value of 3·2 being obtained. This corresponds to an axial ratio of 60 (Svedberg & Pedersen, 1940), or may be interpreted as indicating a high degree of hydration.

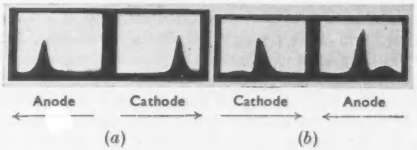

Fig. 1. Electrophoresis patterns of a 2% solution of **A** substance in (*a*) acetate buffer, pH 4·0; $I = 0{\cdot}1$; current, 10·4 ma. Exposure 4 hr. after starting current. (*b*) Phosphate buffer, pH 8·0; $I = 0{\cdot}2$; current 14·5 ma. Exposure 5·25 hr. after starting current.

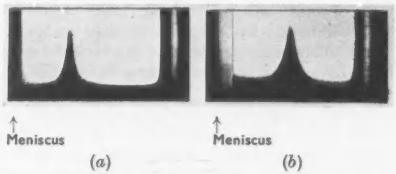

Fig. 2 Sedimentation diagrams of 1% solution of the **A** substance, 60 min. after reaching full speed, 60,000 rev./min. (*a*) Acetate buffer, pH 4·0; $I = 0{\cdot}1 + \text{NaCl}$ (0·15M); (*b*) phosphate buffer, pH 8·0; $I = 0{\cdot}1 + \text{NaCl}$ (0·15 M).

DISCUSSION

The data obtained show that electrophoretically the substance is essentially homogeneous. This is an important finding as it indicates that the nitrogen in the preparation is not attributable to protein contamination, but is an integral part of the molecule.

For a substance having a molecular size of 260,000 associated with the observed large frictional ratio, which is reflected in the concentration dependence of the sedimentation constant, a rather sharp boundary would be expected to be maintained throughout sedimentation, provided that the substance was monodisperse.

The conclusion that the substance is moderately polydisperse must probably be drawn, but the data presented are insufficient to give a precise expression to the range and distribution of molecular size present in the preparation. Another possible explanation of the data might be that the molecules are uniform in mass, but as a result of preparative manipulation have become configurationally deformed to a varying extent.

SUMMARY

1. The blood-group **A** substance has been found to be essentially homogeneous electrophoretically.

2. The mean molecular weight of the substance calculated from diffusion and sedimentation data is 260,000, and the frictional ratio 3·2.

3. The substance is probably moderately polydisperse and the molecular shape highly asymmetrical.

I wish to acknowledge the technical assistance of Mr H. Murray.

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Studies on the Metabolism of Semen

6. ROLE OF HORMONES. EFFECT OF CASTRATION, HYPOPHYSECTOMY AND DIABETES. RELATION BETWEEN BLOOD GLUCOSE AND SEMINAL FRUCTOSE

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The function of the male accessory organs of reproduction is to elaborate the seminal plasma. This activity is subject to a strict control by various hormones, particularly those produced in the testes and the anterior pituitary. Knowledge concerning the relationship between hormones and the functional state of the male accessory organs was provided mainly by anatomical and histological studies. The so-called 'hormone indicator tests' were elaborated from the information gained by these researches. Recently a successful attempt was made to use as indicator tests two chemical reactions, the 'fructose test' and 'citric acid test', based upon the finding that the ability of the accessory glands to produce fructose and citric acid depends upon the activity of the testicular hormone. Both fructose and citric acid were shown to disappear from the seminal plasma as the result of castration and to reappear promptly after implantation or injection of testosterone (Mann & Parsons, 1947; Mann, Lutwak-Mann & Price, 1948; Mann, Davies & Humphrey, 1949).

This paper contains results concerning the quantitative aspects of the relation between testosterone and the secretion of fructose and citric acid in the male accessory organs of reproduction. New findings will be reported, obtained in experiments with hypophysectomized animals, which show that the endocrine influence of the testes on the chemical constituents of semen is integrated with the functioning of the anterior pituitary gland. Another object of the present study was to investigate the part played by yet another hormone, insulin, in the endocrine control of semen composition; experiments will be described, carried out with alloxan-diabetic rabbits, which point to the existence of a definite relationship between the level of fructose in semen and that of glucose in blood (preliminary communication, Mann & Parsons, 1949).

METHODS

In experiments on rabbits, the experimental material consisted of semen and male accessory organs. The collections of semen were carried out at definite time intervals by means of an artificial vagina fitted out with a glass cup receptacle of known weight (Macirone & Walton, 1938). Immediately after ejaculation the glass cup containing the semen was detached from the rubber portion of the vagina and weighed; the amount of the two major components of rabbit semen, gel and fluid, was then quickly measured. Finally the semen was deproteinized by grinding with trichloroacetic acid (final concentration 8% w/v). The accessory organs, obtained by dissection, consisted of glandula seminalis, glandula vesicularis and prostate. Both young and fully mature normal bucks were used. The castration was performed usually so as to remove only the testes, leaving the epididymes intact. Hypophysectomies were carried out in rabbits by Dr G. W. Harris using the technique of Jacobsohn & Westman (1940). Diabetes was produced in rabbits by injecting alloxan intravenously. Only such rabbits were retained for experimental purposes in which the level of seminal fructose before the alloxan treatment was fairly constant, and in which alloxan did not produce toxic symptoms such as loss of weight, fall in sexual drive or decrease in the volume of semen ejaculates. Best results were obtained by giving a rabbit an injection of 75 mg. of alloxan/kg. body weight, and repeating this dose 1 or 2 days later.

In experiments on rats the following organs were used for analysis: seminal vesicle, coagulating gland, ventral prostate and dorsolateral prostate. The rats were killed under ether anaesthesia in order to prevent ejaculation and loss of secretory fluid from the accessory organs of reproduction. Unless otherwise stated in the text, the organs were analysed together with the secretions contained in them. Immediately after dissection the organs were ground with trichloroacetic acid (final concentration 8% w/v) and protein-free extracts obtained by centrifugation. Castration was performed in rats through the scrotum, and the epididymes removed together with both testes.

Fructose was determined colorimetrically; glucose was estimated by means of glucose oxidase, as the difference in the total reducing sugar content before and after aerobic incubation with the enzyme (Mann, 1946, 1948). Citric acid was determined by the method of Speck, Moulder & Evans (1946).

RESULTS

Content of fructose, glucose and citric acid in normal rabbit semen

A single ejaculate of rabbit may vary in volume from less than 1 ml. to as much as 6 ml., but when the two main portions of the semen, gel and fluid, are

measured separately it will be found that it is mainly the quantity of gel which is subject to very considerable variation whereas the fluid portion is more constant. This circumstance has an important bearing on the measurements of fructose and citric acid in rabbit semen. It explains why fructose, which is chiefly associated with the fluid portion, does not vary in quantity from one ejaculate to another nearly as much as citric acid which is found largely in the gel portion. Fig. 1, which represents the record of weekly collections of semen from a single rabbit over a period of 16 weeks, is given as an illustration of this phenomenon. The animal from which this record was obtained was a normal fully mature buck, but with exceptionally large fluctuations in the volume of ejaculates. The volume fluctuated in the same direction as the content of citric acid, with fructose remaining relatively stable. The difference in the behaviour of fructose and citric acid becomes obvious, however, only if the analytical results are considered in terms of absolute quantities (mg. or μg./ejaculate) and not merely as concentrations (mg./100 ml.). However, the content of fructose was by no means equally stable in all the rabbits investigated; it was not unusual to find rabbits with fairly wide day-to-day fluctuations. Considerable individual variations in seminal fructose were established between different rabbits. Parsons (1950) has recently analysed seventy-two ejaculates collected from twelve different normal rabbits; the average values (each representing an average from six ejaculates collected at weekly intervals) covered a range from 280 to 962 μg./ejaculate or 40–420 mg./100 ml. of semen.

The present study has brought to light a new fact, namely, that rabbit semen, unlike that of bull, ram and man, occasionally contained an appreciable, but variable, admixture of glucose in addition to fructose. The glucose content was sometimes low or negligible but some ejaculates contained as much as 400 μg. This fact will be described more fully in connexion with the action of insulin on the composition of semen in diabetic animals (Table 5).

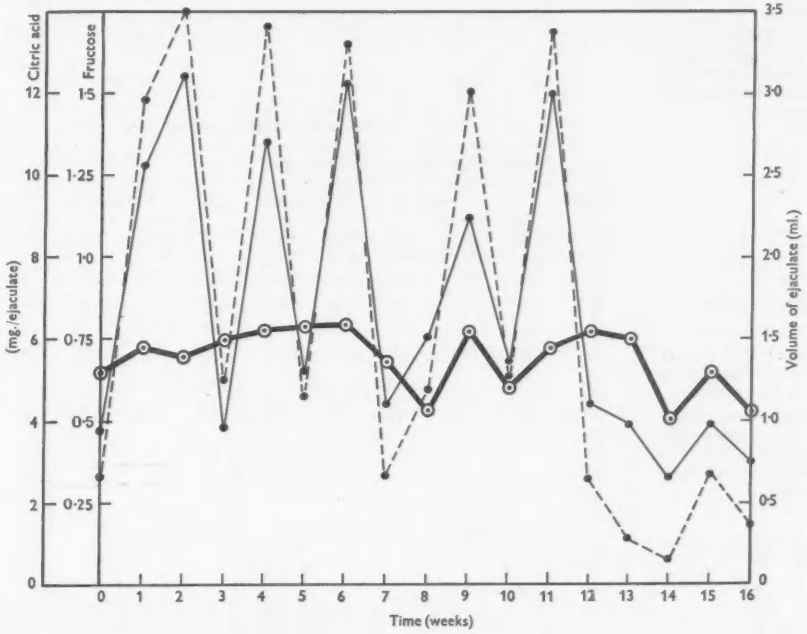

Fig. 1. Relation between volume (●—●—●) of ejaculate and content of fructose (⊙—⊙—⊙) and citric acid ●- -●- -●- in rabbit semen.

Effect of castration and testosterone

The effects of castration and testosterone were studied on rabbits and rats. In rabbits the semen and accessory organs become largely depleted of fructose within 2–3 weeks after castration (Mann & Parsons, 1947); there is also a sharp decline in the amount of gel and citric acid (Humphrey & Mann, 1949). Results presented in Table 1 are the outcome

Table 1. *Effect of castration and testosterone on the semen and accessory organs of reproduction in the rabbit*

	Semen					
	Average from animals (no.)	Volume (ml.)	Fructose		Citric acid	
			(mg./100 ml.)	(μg./ejaculate)	(mg./100 ml.)	(μg./ejaculate)
Normal buck	10	1·3	55	720	210	2750
2 weeks after castration	4	0·4	35	140	80	320
3 weeks after castration	4	0·3	3	9	15	45
6 weeks after castration and simultaneous implantation of testosterone (100 mg.)	4	2·0	34	680	150	3000
6 weeks after castration and simultaneous implantation of testosterone, pellet removed and rabbit examined 3 weeks later	2	0·2	5	10	30	60

	Accessory organs with their secretory fluids										
		Glandulae vesicularis and seminalis					Prostate				
	Average from animals (no.)	Weight (mg.)	Fructose		Citric acid		Weight (mg.)	Fructose		Citric acid	
			(mg./100 g.)	(μg./organ)	(mg./100 g.)	(μg./organ)		(mg./100 g.)	(μg./organ)	(mg./100 g.)	(μg./organ)
Normal buck	4	930	45	420	328	3050	870	120	1040	50	440
2 weeks after castration	2	610	5	32	70	430	520	19	100	3	34
3 weeks after castration	3	480	2	10	18	86	420	3	13	1	4
6 weeks after castration and simultaneous implantation of testosterone (100 mg.)	2	1200	29	350	210	2540	1160	87	1020	38	440
6 weeks after castration and simultaneous implantation of testosterone, pellet removed and rabbit examined 3 weeks later	2	800	4	32	25	200	930	14	130	2	18

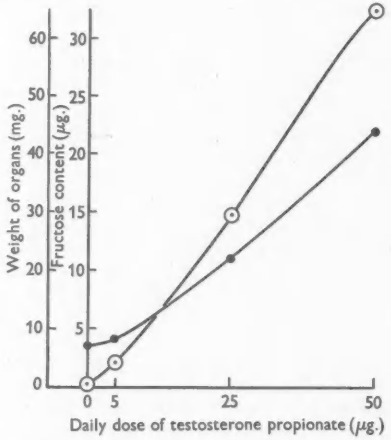

Fig. 2. Dosage-response curves of testosterone propionate, using the coagulating glands of the rat; ⊙—⊙—⊙, fructose (μg.); ●—●—●, weight of organs (mg.).

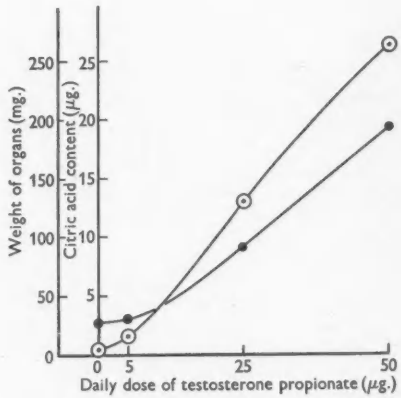

Fig. 3. Dosage-response curves of testosterone propionate, using the seminal vesicles of the rat; ⊙—⊙—⊙, citric acid (μg.); ●—●—●, weight of organs (mg.).

Table 2. *Effect of castration and testosterone on male accessory organs of the rat*

Condition of rats	Average from animals (no.)	Coagulating glands			Dorsolateral prostate			Seminal vesicles			Ventral prostate		
			Fructose			Fructose			Citric acid			Citric acid	
		Weight (mg.)	(μg.)	(mg./100 g.)	Weight (mg.)	(μg.)	(mg./100 g.)	Weight (mg.)	(μg.)	(mg./100 g.)	Weight (mg.)	(μg.)	(mg./100 g.)
Normal:													
1-year old, average weight 430 g.	3	230	293	127	440	170	38	1590	970	57	750	293	39
Castrated when 3 weeks old:													
Autopsied when 1 year old	6	3	0	0	6	0	0	6	0	0	8	0	0
Left for 11 months, then implanted with 100 mg. of testosterone; autopsied 5 weeks later	2	193	402	207	523	345	66	1413	1150	80	334	350	105
Left for 1 year, then injected with testosterone propionate as follows:													
8 injections of 50 μg. daily	2	15	18	120	40	20	50	159	120	75	50	30	60
30 injections of 50 μg. daily	2	40	90	225	90	85	94	320	390	122	178	115	65
Castrated when 11 months old:													
Autospied 7 weeks later	3	30	1	3	75	3	4	135	25	18	83	23	27
Left 4 weeks, then injected with testosterone propionate:													
21 injections of 200 μg. daily	2	260	293	113	470	280	60	1250	680	54	700	320	46

Table 3. *Effect of testosterone propionate on normal rats*

Rats (4 months old)	Weight of rat (g.)	Weight of organs (mg.)						Fructose (μg.)		Citric acid (μg.)	
		Testis (one)	Epididymis (one)	Coagulating glands	Dorsolateral prostate	Seminal vesicles	Ventral prostate	Coagulating glands	Dorsolateral prostate	Seminal vesicles	Ventral prostate
Non-treated	215	932	327	84	129	187	152	64	70	50	29
	215	1026	335	48	152	180	195	64	64	27	63
	230	938	313	37	134	192	245	58	69	37	175
30 injections of 200 μg. daily, prior to autopsy	205	748	354	200	413	1058	474	355	251	305	381
	240	859	436	166	376	1376	585	255	300	870	910

of a combined study of both semen and accessory organs in a group of buck rabbits of similar age (16–22 months) and weight (2·7–3·2 kg.); they show the close dependence between the male sex hormone and the secretory function of the reproductive organs, as expressed in the fructose and citric acid content. Testosterone implanted immediately after castration prevented the post-castration fall of fructose as well as of citric acid, but when the implant was removed there was a prompt fall in the level of both substances. On the other hand, the post-castration reduction in the volume of the ejaculates, and in the weight of the reproductive organs, were not nearly as large or rapid as the change in the output of fructose and citric acid.

In rats the response to castration and testosterone is essentially the same as in rabbits. Much depends, however, on the age of the rats at castration, the period allowed for post-castration regression, the length of time during which the hormone treatment is continued and the method of application (implantation or injection); allowance should also be made for considerable individual variations in the response to testosterone even in rats of the same age and strain.

The following experiment was set up to study the relationship between the daily injected dose of testosterone propionate and the level of fructose and citric acid in the rat accessory organs. Twenty-six rats were castrated when 7 weeks old, left for 8 weeks, and then 4 groups of 8, 8, 6 and 4 animals injected daily for 3 weeks with 0, 5, 25 and 50 μg. of testosterone propionate respectively. At autopsy the material dissected from rats of the same group was pooled, weighed, deproteinized, analysed, and the results calculated in μg. of fructose and citric acid per animal. Figs. 2 and 3 illustrate the results obtained with two organs, the coagulating gland and the seminal vesicle. These two organs were chosen because it was previously shown by Humphrey & Mann (1949) that in the rat, fructose is formed in the coagulating gland, citric acid in the seminal vesicle. The results for each gland are expressed by two dosage-response curves obtained by plotting the arithmetic dose of the hormone against the weight of the organ and the contents of fructose and citric acid respectively. As can be seen from both Figs. 2 and 3, the chemical secretory response to testosterone was proportionally much greater than the hormone-induced increase in weight of organs. For instance, the smallest dose of hormone, 5 μg. of testosterone propionate per day, caused in the castrated rats a rise in fructose and citric acid from 0·3 to 2·0 μg. and from 0·4 to 1·5 μg. respectively, whereas the corresponding increases in weight were from 7 to 8 mg. in the coagulating glands, and from 26 to 29 mg. in the seminal vesicles.

The dependence of the activity of the accessory glands upon the male sex hormone was further demonstrated on rats which were castrated when they were only 3 weeks old, that is before their accessory organs began to produce either fructose or citric acid. In such rats, left untreated for a year, the accessory organs rapidly responded to treatment with testosterone as shown by the amounts of fructose and citric acid secreted by them (Table 2).

Normal, 4-month-old rats, injected with massive doses of the male hormone (200 μg. of testosterone propionate daily) responded by increasing the level of fructose and citric acid formation above that of the normal non-treated controls (Table 3). However, when the injections were extended, the state of overstimulation in the accessory organs was accompanied by a marked decline in the size of the testes; after 7 weeks' treatment with 200 μg. of testosterone propionate per day the reduction in the weight of testes was nearly 50 %.

Effect of hypophysectomy and gonadotrophin

Hypophysectomy produced effects similar to castration. The hypophysectomized rabbit responded to the subcutaneous implantation of a pellet of testosterone (100 mg.) with renewed secretion of fructose and citric acid. When, instead of testosterone, the pregnant-mare serum gonadotrophin ('Gestyl', Organon) was used, the accessory organs responded in a similar manner (Table 4). It was interesting to note that the effect obtained with gonadotrophin was more pronounced with regard to citric acid (glandula vesicularis) than fructose (prostate). However, it would be necessary to confirm this observation on a larger number of experimental animals before it could be considered significant.

Relationship between glucose in blood and fructose in semen

It was found in an earlier experiment that when a normal animal was rendered severely hypoglycaemic with insulin for a short period of time (11 hr.), the content of fructose in accessory glands was not markedly changed (Mann *et al.* 1949). Clearly, to tackle the problem of the relationship between blood glucose and seminal fructose it was essential (1) to establish experimental conditions which would ensure not merely transient but more lasting increases or decreases in the level of blood sugar, and (2) to carry out a sufficiently large number of semen collections and fructose analyses in order to exclude results due to normal fluctuations. To satisfy these requirements several methods were tried, but the most satisfactory results were obtained in the following manner. The extent of normal variations of seminal fructose in a given animal was first established in a series of estimations, then the animal was rendered diabetic with alloxan, and the estimations

of blood sugar and seminal fructose continued for several more weeks or months. Fig. 4 illustrates the course of such an experiment performed on a rabbit from which regular collections of blood and semen of individual ejaculates, as can be seen, for instance, from Table 5. As a matter of fact, however, in the case of rabbit-seminal fructose both methods of presentation, concentration (mg./100 ml.) and con-

Table 4. *Influence of hypophysis on the formation of fructose and citric acid in male accessory organs*

Buck rabbit	Glandula vesicularis			Prostate		
	Weight (mg.)	Fructose (μg./ organ)	Citric acid (μg./ organ)	Weight (mg.)	Fructose (μg./ organ)	Citric acid (μg./ organ)
1. Normal	930	864	2840	810	880	430
2. 4 weeks after hypophysectomy	159	24	40	149	10	6
3. 4 weeks after hypophysectomy and simultaneous implantation of testosterone	1190	208	840	1360	930	410
4. 6 weeks after hypophysectomy; for the last 4 weeks injected with 200 i.u. Gestyl* daily	1140	165	8280	526	210	395
5. 4 weeks after castration	350	5	15	280	20	10
6. 4 weeks after castration and simultaneous implantation of testosterone	1200	730	2180	1080	1350	210

* Pregnant-mare serum gonadotrophin, Organon.

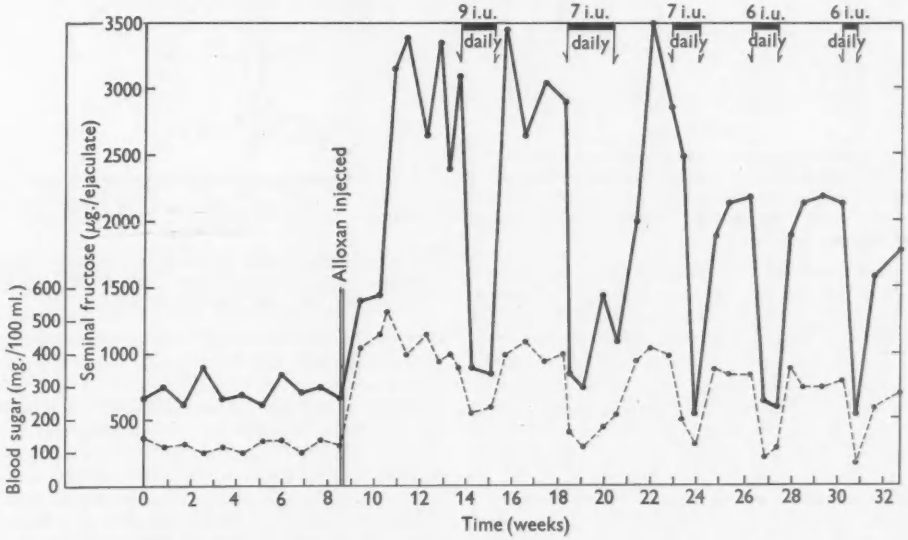

Fig. 4. Effect of alloxan diabetes and insulin on seminal fructose in the rabbit. The periods of insulin treatment and daily dose (in units, i.u.) are indicated by arrows; ●—●—●, seminal fructose, ●- -●- -●, blood sugar.

were carried out during a period of 7 months. It may be noted in Fig. 4 that whereas the blood sugar values are expressed in mg./100 ml., those relating to fructose in semen are given in μg./ejaculate and not in terms of concentration. This method was adopted because it brings out more clearly the quantitative difference in the chemical composition tent (μg./ejaculate), yield similar curves so long as the concentration of fructose in semen is given in mg./100 ml. of the fluid portion of the semen and not of the whole semen, which includes variable amounts of gel. In the buck used for the experiment illustrated in Fig. 4 the volume of the fluid portion varied not more than from 0·8 to 1·2 ml. This animal had,

before the alloxan injections, a blood sugar content of 100 mg./100 ml. and of fructose in semen about 700 μg./ejaculate or 70 mg./100 ml.; as a result of alloxan treatment this rabbit developed glucosuria and its blood sugar level rose to 400–550 mg./100 ml. At the same time there was an increase in seminal fructose which reached a peak value of 3000 μg./ejaculate or 300 mg./100 ml. at about 3 weeks after the injections of alloxan. When it was satisfactorily established that repeated collections of semen from the diabetic animal continue to yield ejaculates with an abnormally high fructose content, a study was commenced of the effect of insulin on the diabetic animal. Insulin was injected three times daily in

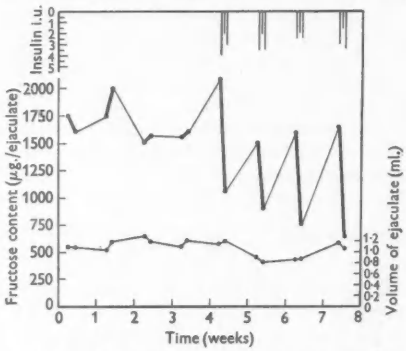

Fig. 5. Effect of insulin treatment of short duration on seminal fructose in the diabetic rabbit. Collections of semen made on the first and second day of every week: upper curve, ●—●—●, fructose; lower curve, ●—●—●, volume.

quantities sufficient to bring the blood sugar level down to 100–200 mg./100 ml. within 2 hr. after each injection. For this purpose relatively large doses of insulin were required at first, up to 10 units/day. A few weeks later, however, when the treatment was repeated, the same effects could be produced with smaller doses of insulin. As regards the behaviour of fructose, it can be seen from Fig. 4 that simultaneously with the insulin-induced fall in blood glucose, there was always a big reduction in the fructose content of semen, and that when the effect of insulin on blood glucose wore off, fructose in semen also rose.

In order to find out how soon after insulin administration the level of seminal fructose is affected, the following experiment was performed with an alloxan-diabetic rabbit (Fig. 5). For 8 weeks in succession, two collections of semen were made each week on the first and second day respectively. Without insulin the two ejaculates on the two successive days differed very little from each other with respect to volume or fructose content. However, if the rabbit was subjected to treatment with insulin immediately after the first collection, then the next day the fructose content of semen was considerably lowered although the volume of semen remained unchanged.

In another experiment two littermate buck rabbits were used; one was rendered diabetic with alloxan, the other remained as untreated control. The experiment summarized in Fig. 6 shows that alloxan diabetes has produced a very distinct increase in the output of fructose in semen. In this particular experiment, however, the degree of variation in the fructose content was much more pronounced in the

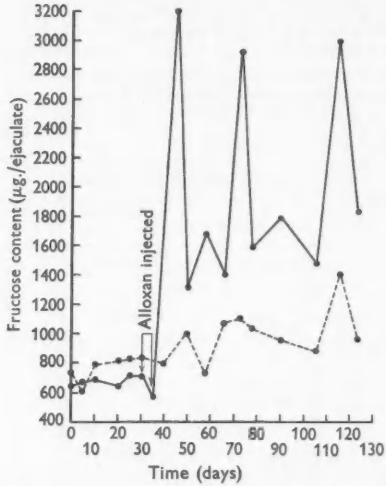

Fig. 6. Fructose content of semen in littermate rabbits, normal and alloxan-diabetic; ●—●—●, diabetic rabbit; ●- - -●- - -●, control littermate.

diabetic buck than in the control littermate, and occasionally the diabetic animal yielded ejaculates with a nearly normal sugar content.

The level of citric acid, unlike that of fructose, was not markedly affected by alloxan diabetes or by insulin. On the other hand, the level of seminal glucose was definitely increased in diabetic rabbits and it responded to insulin treatment in a manner similar to fructose. This can be seen from Table 5 which offers also a comparison between the level of glucose in blood and that of fructose in semen. It is essential to point out, however, that the results recorded in Table 5 were based on analyses of ejaculates collected at about 3-day intervals. Different results would have been obtained if the semen collection had been more frequent. Table 6 records several experiments (*A* 1, *B* 1, *C* 1, *D*, *E*, *F* and *G*) in which ejaculates were obtained both from normal and from diabetic rabbits at very short and frequent time intervals. From these it can be seen

that as a result of frequently repeated semen collections the citric acid level came down very considerably; moreover, the depletion of citric acid went further in the diabetic than in the normal control animal. It may also be noted from Table 6 that, due to great frequency in semen collections, there was a much sharper decline in the volume of semen and its fructose content in the diabetic than in the normal 1180 mg./100 ml., and 40·6, 37·6, 35·3 and 42·6 mg./ejaculate. Harvey (1948), in her recent survey of fructose in 150 specimens of human semen, recorded values ranging from 5 to 640 mg./100 ml. or 0·05 to 31·6 mg./ejaculate. Even if due allowance is made for the very large variations in the fructose level of normal human semen, the fructose values found in the diabetic semen doubtless exceed the normal level.

Table 5. *Effect of diabetes and insulin on the content of fructose, glucose and citric acid in rabbit semen*

	Semen							
		Fructose		Glucose		Citric acid		Blood sugar
Days (no.)	Ejaculate (ml.)	(μg./ ejaculate)	(mg./ 100 ml.)	(μg./ ejaculate)	(mg./ 100 ml.)	(μg./ ejaculate)	(mg./ 100 ml.)	(mg./ 100 ml.)
1	0·65	635	97	210	32	3850	587	132
4	0·16	630	400	120	78	670	425	134
7	2·07	740	36	0	0	12100	584	116*
10	1·42	760	54	42	3	456	32	125
13	Injected intravenously alloxan (75 mg./kg.)							
14	Injected intravenously alloxan (75 mg./kg.)							
17	0·67	1700	253	310	46	3650	545	485†
20	1·24	3540	285	780	63	13200	1064	492
23	0·63	1280	205	137	22	3020	479	272
26	3·03	3280	108	1665	55	14100	465	434
29	1·29	2980	231	890	69	4040	313	340
30–33	Injected daily with 10 units of insulin							
34	3·20	790	25	10	0·3	15800	493	135
37	1·05	3450	329	650	62	12100	1152	515
40	3·80	1790	47	460	12	29100	765	425
41–43	Injected daily with 8 units of insulin							
44	1·09	850	77	0	0	7000	642	149

* Glucose (89 mg. as estimated by glucose oxidase) + 2 mg. of fructose (colorimetrically) + 26 mg. of reducing, non-fermentable material.

† Glucose (469 mg.) + 2 mg. of fructose + 14 mg. of reducing, non-fermentable material.

rabbit. These effects must be attributed to the generally lower rate of semen regeneration in diabetic individuals than in healthy normal males.

Several experiments were carried out to investigate the effect of intravenously introduced substances such as glucose, fructose, sucrose and Na pyruvate, upon the rate of fructose generation in the male reproductive organs. In these experiments, however, with the exception of fructose itself, none of the injected substances affected the output of fructose in semen. However, even in the case of fructose one must not overlook the possibility that the observed increase in seminal fructose may have been at least partly due to general flooding with fructose of tissues and body fluids, as shown by the excretion of a fairly large proportion of the injected fructose through the kidneys.

Although our results concerning the relationship between blood glucose and seminal fructose have been so far confined to rabbit, it appears that similar conditions also prevail in man. In four samples of semen from three diabetic patients the following values for fructose were found: 765, 940, 820 and

DISCUSSION

The composition of seminal plasma, unlike that of other body fluids, is subject to considerable fluctuations, even in the same animal. As a mixture of secretions produced by several accessory organs of reproduction, the seminal plasma varies according to the relative contribution made by the organs concerned. In this connexion the actual size and 'storage' capacity of each of these glands are, of course, of great importance. Another determining factor is the influence of the male sex hormone upon the capacity of the accessory glands to produce characteristic constituents of semen, such as fructose and citric acid (Mann & Parsons, 1947; Humphrey & Mann, 1949). Based on this observation the estimation of fructose and citric acid were elaborated into a 'hormone indicator test' of considerable sensitivity and at the same time of great simplicity. It was shown earlier by Mann *et al.* (1949) that in castrated bull calves the early effects of an implantation of testosterone can be recognized much more easily through the large increase in the content

Table 6. *Effect of intravenously injected carbohydrate on the content of fructose and citric acid in semen*

Rabbit	Exp. no.	Injected substance	Time after injection (hr.)	Semen: Ejaculate (g.)	Semen: Fructose (μg./ejaculate)	Semen: Fructose (mg./100 g.)	Semen: Citric acid (μg./ejaculate)	Semen: Citric acid (mg./100 g.)	Urine. Sugar excreted in 24 hr.	Blood. Reducing sugar (mg./100 ml.)
A (normal)	1	None	0	1·48	1080	73	7830	535	—	—
			2	0·64	1280	200	400	62	—	—
			6	0·59	450	76	250	42	—	—
			24	1·26	780	62	2150	170	—	—
	2	Glucose (10 g.)	0	1·09	980	90	5700	522	Glucose, 0·7 g.	124
			2	0·40	900	224	287	72		238
			6	0·51	1230	241	610	120		134
			24	1·19	870	73	1230	103		138
B (normal)	1	None	0	3·25	1210	37	16350	500	—	—
			2	1·00	1420	142	520	52	—	—
			6	0·30	1080	360	520	173	—	—
			24	1·66	630	38	3780	227	—	—
	2	Sucrose (12 g.)	0	2·51	1060	42	20200	800	—	136
			4	0·40	780	194	450	112	—	158
	3	Fructose (10 g.)	0	0·90	1090	121	3150	350	Fructose, 1·2 g.	130
			4	0·30	3250	1083	215	71		216
			24	0·31	760	245	430	139		125
	4	Fructose	0	2·04	1190	59	11600	570	Fructose, 1·3 g.	—
			4	0·90	8610	957	515	57		—
			24	0·76	1570	206	2450	—	—	—
C (diabetic)	1	None	0	1·44	2960	206	10500	322	Glucose, 9 g.	385
			4	0·50	1130	226	1060	212		410
			24	0·98	795	81	1150	118		360
	2	Glucose (10 g.)	0	3·04	1920	63	15400	502	Glucose, 16 g.	224
			4	1·00	1150	115	1750	175		231
			24	1·40	520	37	6500	465		385
	3	Fructose (10 g.)	0	3·30	2450	74	11200	370	—	365
			4	1·21	2700	223	1950	161	—	382
			24	1·60	1080	67	3700	231	—	333
	4	Na pyruvate* (2 g.)	0	1·41	1940	137	4640	330	—	—
			4	0·55	610	111	900	164	—	—
			24	1·39	1020	73	4750	342	—	—
D (diabetic)	1	None	0	1·71	2300	134	13100	766	—	419
			2	0·50	1285	257	450	90	—	—
			6	0·10	20	20	52	52	—	385
E (normal)	1	None	0	3·09	875	28	3310	107	—	129
			2	1·28	778	61	1640	128	—	—
			6	0·72	540	75	750	104	—	134
F (normal)	1	None	0	2·45	1210	49	11600	474	—	—
			2	0·95	1700	179	515	54	—	—
			6	1·03	780	75	1115	111	—	—
G (diabetic)	1	None	0	0·89	1880	210	8650	970	—	—
			2	0·39	620	160	890	228	—	—
			6	0·10	20	20	20	20	—	—

* The 3 ejaculates contained 0, 41 and 154 μg. of pyruvic acid, respectively.

of fructose and citric acid in the seminal glands than the rather insignificant histological recovery changes in the same glands. An example of the intimate relationship between male hormone and the secretion of fructose and citric acid was also provided by experiments in which rat accessory gland tissues completely separated from the rest of the male reproductive tract and transplanted subcutaneously into male or female hosts responded readily to testosterone by producing fructose and citric acid (Mann *et al.* 1948; Lutwak-Mann, Mann & Price, 1949). The determination of fructose and citric acid was also made use of to define the degree of androgenic activity even when this activity is relatively slight; thus, for instance, it has been applied successfully to the study of the androgenic activity of intact ovaries,

and of pure progesterone in rat (Price, Mann & Lutwak-Mann, 1949).

In the present study of the relationship between the testicular hormone and the chemical processes of secretion in the accessory organs, the quantitative aspects have been examined. The experiments with castrated rats receiving small doses of testosterone propionate, 5–50 μg./day, showed that the changes in the level of fructose and citric acid are much more prominent than the hormone-induced increases in size and weight of the corresponding rat organs. There are, of course, other sensitive androgenic tests such as, for instance, the capon comb test; however, results obtained with the capon test have no direct bearing on the problem of the quantitative relationship between the action of testosterone and the elaboration of seminal plasma in the male accessory organs, particularly in mammals. The fructose and citric acid tests are applicable to all species which normally secrete fructose and citric acid in semen, such as bull, ram, boar, stallion, guinea pig, rabbit and rat. On the other hand, they cannot be made use of in the capon since cock semen does not contain fructose or citric acid (Mann, 1948).

In a discussion of the significance of the testicular hormone as a factor which influences the composition of the seminal plasma, it is essential to bear in mind that in the living animal the activity of the male sex hormone is not solely dependent on the capacity of the testes to produce the hormone, but is in addition influenced indirectly by the action of the anterior pituitary hormones on the testes. In this paper hypophysectomy has been shown to be as effective as gonadectomy in abolishing the formation of fructose and citric acid in the accessory organs. It was possible to restore with gonadotrophin (from pregnant-mare serum) the fructose and citric acid formation in hypophysectomized rabbits.

Apart from the testes and the anterior pituitary, yet another organ, the pancreas, has now been shown to influence one seminal component, namely fructose. This influence is an indirect one: it is exerted by insulin on the level of fructose in semen. The link-up between the blood sugar level and seminal fructose has been studied in rabbits rendered diabetic by means of alloxan. Experimental diabetes caused a distinct rise in the level of fructose in semen. It was noticed, however, that the peak values were attained only after the hyperglycaemia has prevailed in the rabbit for some time. It is conceivable that some additional agents or mechanisms are involved in establishing the blood glucose:seminal fructose relation. Experiments on rabbits have shown that in severe diabetes the level of fructose in semen may exceed 4–5 times the control values, and that it can be brought back to normal with suitable doses of insulin. The rapid response to insulin, at any rate in the rabbit, was demonstrated by experiments in which collections of semen were taken shortly before and one day after, insulin treatment which led invariably to a sharp decline in the content of seminal fructose.

The behaviour of seminal fructose in diabetes must be taken as a strong indication that blood glucose acts as precursor of seminal fructose. The experiments on diabetic rabbits also lend new significance to the observations previously reported by Mann & Lutwak-Mann (1948) that small amounts of fructose are formed *in vitro* from glucose in the presence of minced seminal gland of bull. The conclusions drawn from experiments on rabbits probably apply also to other species, including man, where abnormally high values of seminal fructose were found to be associated with diabetes. However, the fact must not be overlooked that there are species such as, for example, sheep, where the normal blood sugar level is notoriously low, and which nevertheless exhibit normally a high level of fructose in semen.

SUMMARY

1. The formation and composition of seminal plasma is subject to control by several hormones.

2. The withdrawal of the male sex hormone through castration results in disappearance from the seminal plasma of two characteristic components, fructose and citric acid. The formation and secretion of both these substances can be successfully re-established by subcutaneous implantation or injection of pure testosterone.

3. A direct relationship exists between the amount of testosterone injected into castrated rats and the response of the accessory organs to produce fructose and citric acid. This makes it possible to use the quantitative assay of fructose and citric acid as a sensitive and simple 'hormone indicator test'.

4. Hypophysectomy produces effects similar to castration. The secretion of fructose and citric acid by the organs of the hypophysectomized animals can be induced by the administration of either gonadotrophin or testosterone.

5. In diabetes the content of fructose in semen is considerably increased. In alloxan-diabetic rabbits it was possible to demonstrate the existence of a relation between hyperglycaemia and the fructose level in semen. Insulin administration causes a fall in the level of fructose in the semen of diabetic rabbits.

6. The evidence is discussed which indicates that glucose acts as a precursor of seminal fructose in the animal body.

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A Note on the Copper-retaining Power of a Humic Acid from Peat Soil

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Bremner, Mann, Heintze & Lees (1946) have suggested that trace elements may be retained in soil as metallo-organic complexes with the soil organic matter. Lees (1948) estimated, from results obtained with whole cacao soils, that the copper retention is about 1400 μequiv. copper/g. soil organic matter. The copper-retaining power of 'humic acid' (the fraction of soil organic matter to which most of the trace element retention is presumably due) has now been measured directly.

EXPERIMENTAL

A peat soil from the San Juan Estate, Trinidad, was air-dried and a 100 g. sample repeatedly extracted with 500 ml. lots of 0·1 M-sodium oxalate at pH 7·0. Each lot of extractant was shaken with the soil intermittently over a period of 8 hr. after which the soil was allowed to settle for 16 hr.; the extract was then decanted and a fresh 500 ml. added to the residual soil. After five such extractions the combined extracts were acidified with conc. H_2SO_4 to a pH of approximately 2. The humic acid thus precipitated was centrifuged and then redissolved in 250 ml. water plus sufficient solid $NaHCO_3$ to bring the pH of the solution to 7·0. The humic acid solution was then filtered to remove clay and dialysed against repeatedly changed distilled water to remove salts. The humic acid was then reprecipitated by the addition of 4 vol. of 95% (v/v) ethanol and again filtered off. The precipitate was washed repeatedly with ethanol to remove as much water as possible and dried to a black cake in a desiccator over $CaCl_2$. This preparation of humic acid is based on the work of Bremner & Lees (1950).

A 1% aqueous solution of dried, powdered, humic acid was treated in 1 ml. lots with 1 ml. 0·5 M-$Ca(NO_3)_2$ solution (to precipitate the humic acid and to saturate its base-exchange groups) plus 1·0, 1·5, 2·0, 2·5, 3·0 or 3·5 ml. $CuSO_4$ solution containing 10 μequiv. Cu/ml. solution, with sufficient distilled water to bring the final volume to 11 ml. After standing for 2 hr. the suspended humic acid was centrifuged and the Cu in the supernatant liquid was determined by the method of Sherman & McHargue (1942). The Cu retained by the humic acid was calculated by difference. The results obtained by the method are given in Fig. 1.

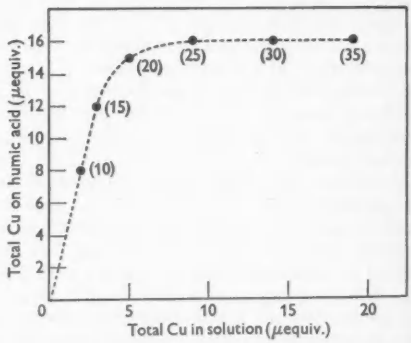

Fig. 1. The distribution of copper (added as $CuSO_4$) between 10 mg. humic acid and 11 ml. of $4·5 \times 10^{-2}$ M-$Ca(NO_3)_2$. (Each point is the mean of three determinations. Bracketed figures show total amount of copper (μequiv.) present.)

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RESULTS AND DISCUSSION

The maximum copper retaining power of the humic acid tested, 1600 μequiv. copper/g. humic acid, agrees reasonably well with the figure of 1400 μequiv./g. obtained indirectly on whole soils (Lees, 1948). Humic acid contains about 56% carbon (Page & duToit, 1932); therefore some 60 carbon atoms are associated with one copper atom in the fully saturated complex of copper and humic acid.

SUMMARY

1. The copper-retaining power of a humic acid from a Trinidad peat soil was 1600 μequiv. copper/g. humic acid. This is in agreement with previous results.

2. The atomic ratio of carbon:copper in the complex so formed is about 60:1.

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Some Properties of Specific Cholinesterase with Particular Reference to the Mechanism of Inhibition by Diethyl *p*-Nitrophenyl Thiophosphate (E 605) and Analogues

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A full account of the properties of substances inhibiting cholinesterase has been given in a recent review by Koelle & Gilman (1949). In the following work the properties of certain organic esters of phosphoric acid which are powerful inhibitors of cholinesterase are described. In particular, the kinetics of the inhibitor-enzyme system have been worked out and certain conclusions have been drawn as to the mode of action of these inhibitors. The properties of a convenient preparation of cholinesterase which has been used for this work are given below.

METHODS

For the cholinesterase preparation blood was taken from a goat into a flask containing 2 ml. of 30% (w/v) Na citrate per 60 ml. of blood, and was stored in the refrigerator at 4°. Washed cells lose their activity on storage. Prior to use in an experiment, the blood was centrifuged, the plasma removed and the cells washed three times with isotonic saline. They were then resuspended in buffer and diluted roughly to the original volume of blood taken.

Determination of cholinesterase activity was carried out using the method of Nachmansohn & Rothenberg (1945). The final concentrations in the Warburg flask, after tipping in the contents of the side arm, were NaCl, 0·15 M; $MgCl_2$, 0·035 M; $NaHCO_3$, 0·0312 M and acetylcholine chloride 0·0138 M. The pH of this buffer mixture, gassed with 5% CO_2 in N_2, is 7·72 (calculated from Henderson-Hasselbach equation). Stock salt solutions of 20 times the strength used in the preparation of the buffer were stored in the refrigerator in paraffin-waxed flasks. The buffer was likewise stored after dilution from these stock solutions. The total volume of fluid in each flask was 4·0 ml. except where otherwise stated. All measurements of red-cell suspensions were made with a Krogh-Keys syringe pipette. The contents of the flasks were gassed in the bath at 37° with a slow stream of 5% CO_2 + 95% N_2 for 10 min., and allowed to equilibrate for 10 min. After the addition of the acetylcholine from the side-arm, readings were taken every 10 min., for 1 hr. After a visual inspection of the readings to see that a straight line relationship between time and output of CO_2 was sensibly satisfied, the slope of the line (output in μl. of CO_2/min.) was determined by the method of least squares (Aldridge, Berry & Davies, 1949). The flasks were cleaned after each experiment by boiling in soap solution to remove all lanoline grease and protein, and once a day were further treated with ethanol and HNO_3, rinsed in distilled water and dried in a warm oven.

E 605 and its analogues, the formulae of which are given below, were dissolved initially in absolute ethanol (1 mg./ml.) and were diluted with buffer from that solution prior to the experiment. All the inhibitors were stored at 4° in the dark in bottles with stoppers sealed with paraffin wax.

Throughout this paper their code numbers (given in brackets) are used. They are all pale yellow liquids when pure, but both E 605 and Q 2 darken on standing, particularly when exposed to light. They all hydrolyse slowly in water to give a substituted phosphoric acid and *p*-nitrophenol or

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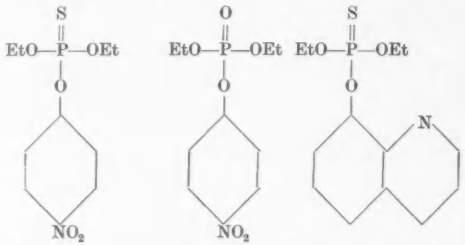
8-hydroxyquinoline. Their stability increases in the order E600, E605 and Q2. E600 is the most soluble in water (approx. 2 mg./ml.), while E605 and Q2 dissolve to the extent of approx. 20 and 40 μg./ml. respectively at 37°. Other inhibitors used were di-*iso*propyl fluorophosphonate (DFP), bis(β-chloroethyl)-*N*-methylamine hydrochloride (N mustard) and eserine sulphate.



p-Nitrophenyl diethyl thiophosphate (E605) | *p*-Nitrophenyl diethyl phosphate (E600) | 8-Quinolyl diethyl thiophosphate (Q2)

RESULTS

Red cells as a specific cholinesterase preparation. It has been shown by Paléus (1947), Brauer & Root (1945) and Augustinsson (1948) that the specific cholinesterase of red cells is attached to the red-cell membrane. It was therefore thought that intact red cells would provide a suitable preparation of cholinesterase for work with inhibitors; intact red cells afford a cleaner preparation than haemolysed cells, and it is also possible to wash them by centrifugation after inhibition of their cholinesterase and so demonstrate reversibility under appropriate conditions. Goat red cells have been used for two reasons: (1) goat serum and red cells contain little or no pseudo-cholinesterase as estimated by ability to split benzoylcholine (Mendel, Mundell & Rudney, 1943); and (2) it is possible to use the same animal repeatedly as a source of red cells. By this means it has been possible to use the activity of the fresh samples of blood as a rough check on the method; since the cholinesterase level of goat red cells from the same animal seems to remain constant for some months. The need for constant rechecking of the method and care in cleaning glass ware need no emphasis when working with such active inhibitors.

Table 1. *Properties of intact and haemolysed red cells*

Concentration of inhibitor (M)	Activity (μl. CO_2/min.) Intact red cells	Haemolysed red cells
Nil	4·25	4·29
$1·6 \times 10^{-8}$ E600	2·34	2·42
$6·9 \times 10^{-7}$ eserine sulphate	2·74	2·59

The results in Table 1 not only confirm the finding of Augustinsson (1948) that the activity of haemolysed cells is the same as that of the intact cells, but also show that both are inhibited to an equal degree by the same concentration of inhibitor.

Activity of inhibitors. For the purposes of comparison with other work 50% inhibition values are given. Because the inhibition is progressive it is essential that the times of incubation are clearly stated when inhibition values are expressed. For this reason double-armed Warburg flasks have been used, one arm for the inhibitor, the other for the substrate, and the centre well containing the red-cell suspension in buffer. In this way the time and temperature of incubation are accurately known.

Table 2. *Concentrations for* 50% *inhibition*

(Incubation time, 30 min., 37°)

Inhibitor	Concentration for 50% inhibition (M)
E605	$1·26 \times 10^{-6}$
E600	$2·14 \times 10^{-8}$
Q2	$3·16 \times 10^{-5}$
N mustard	$9·44 \times 10^{-5}$
Eserine	$1·4 \times 10^{-6}$
DFP	$4·6 \times 10^{-7}$

The values given in Table 2 have been determined by the conventional method of plotting percentage inhibition against the negative log of the M-concentration of inhibitor. Sigmoid curves are obtained. As will be explained later, a better way for irreversible inhibitors is to plot concentration of inhibitor against log percentage residual activity when straight lines are obtained.

Irreversibility of DFP, E600 and E605. Many investigators have shown eserine and prostigmine to be reversible inhibitors by the simple procedure of dialysing the cholinesterase preparation after inhibition, whereby the activity is rapidly regained. It can also be shown that if cholinesterase is first incubated with eserine and acetylcholine is then added to determine the residual activity a curve convex upward is obtained on plotting output in μl. of CO_2 against time. This can only be due to replacement by acetylcholine of physostigmine molecules on the active groups of the cholinesterase. This phenomenon may be considered as an indication of reversibility though not necessarily so. Benzoylcholine has been shown to be a competitive inhibitor of true cholinesterase (p. 457). Table 10 gives the inhibition data for benzoylcholine at the usual concentration of acetylcholine (0·0138M). If cholinesterase is incubated with benzoylcholine and the acetylcholine then tipped in, the output of carbon dioxide is linear with respect to time over the whole range, even though readings are taken every 3 min. This indicates that although benzoylcholine is a

reversible inhibitor of true cholinesterase, an equilibrium is reached between acetylcholine and benzoylcholine so quickly that no changes in rate of output of carbon dioxide may be determined. The difference between eserine and benzoylcholine is presumably one of rate of combination.

Washing intact red cells after inhibition has also been examined as a possible demonstration of reversibility. Table 3 gives the results obtained when red cells inhibited with eserine are centrifuged and washed a varying number of times. The activity is readily recovered, as is also shown for benzoylcholine in Table 12.

Table 3. *Use of intact red cells to demonstrate reversibility*

(Red cells first incubated in buffer containing $8{\cdot}8 \times 10^{-6}$ M-eserine)

Further treatment	Original activity (%)
None	15
Centrifuged, resuspended in buffer	56
Centrifuged, washed twice	95

Studies on the reversibility of DFP by dialysis of the inhibitor-enzyme mixtures have given negative results (Mackworth & Webb, 1948). DuBois, Doull, Salerno & Coon (1949) claim that the inhibition of rat-brain cholinesterase by E 605 is readily reversible *in vivo*. Using the washing technique described above, we have failed to demonstrate any reversibility of E 605 (Table 4).

Table 4. *Irreversibility of E 605 by washing technique*

($1{\cdot}96 \times 10^{-6}$ M-E 605 incubated with red cells (ice cold) for 15 min.)

Treatment	Final activity as percentage of original activity
Uncentrifuged and determined immediately	80
Centrifuged, washed once	84
Centrifuged, washed twice	82
Centrifuged, washed three times	80
Centrifuged, washed four times	84

Nachmansohn, Rothenberg & Feld (1947) have shown a considerable degree of reversibility of DFP inhibition of cholinesterase from both electric tissue of the eel and from caudate nucleus of the ox. The principle of their 'dilution technique' was to incubate cholinesterase with a certain concentration of DFP and at various times to take identical samples, dilute one with the same concentration of DFP in buffer (U = undiluted with respect to DFP) and the other with buffer alone (D = diluted with respect to DFP). If the inhibition is reversible there should be some reactivation of the enzyme in the one diluted with buffer (D). For such a technique to give reliable results it is imperative that the reaction between cholinesterase and DFP is stopped at the time of dilution, otherwise, since this inhibition of cholinesterase is progressive (Nachmansohn, Rothenberg & Feld, 1948), the inhibition will continue at its original rate, while that in D will be very much slowed down by the dilution. Therefore, unless this progressive inhibition is stopped, a difference between the activities of U and D will always be obtained which will indicate 'reversibility'. Table 9 shows that, if acetylcholine is present with the enzyme before the inhibitor is added, little or no inhibition is obtained, and this compound has therefore been used to stop the reaction. The ratio of acetylcholine to inhibitor must be high enough to stop the reaction completely, and this can be checked for each experiment as will be described later. Nachmansohn *et al.* (1947) give no precise information when the acetylcholine was added to the cholinesterase preparation; however, for their experiments on electric tissue they state that 'the time required for taking the solution out of the thermostat, diluting it, putting it into the Warburg vessel, gassing and putting back into the thermostat was kept at the minimum necessary and did not exceed 6–8 min.' In their experiments on cholinesterase from ox caudate nucleus a further dilution was made so that it must be assumed that the acetylcholine was not added until at least 8 min. after the dilution; the inhibition in U was therefore progressing at a rapid rate, while that in D would be much slowed down. That the rate was high in U in the experiments of Nachmansohn *et al.* (1947) with caudate nucleus is shown by the fact that at zero time on their graph U shows approximately 75 % inhibition.

A method has been devised which eliminates these sources of error. In tube A are placed 5 ml. of red-cell suspension in buffer, and in tube B 5 ml. of buffer containing inhibitor (concentration $2x$ μg./ml.). Both tubes are stored in ice. Into the main compartment of one single-armed Warburg flask (U) are pipetted 4·5 ml. of buffer containing x μg. of inhibitor/ml. together with the correct concentration of acetylcholine, and into another Warburg flask (D) 4·5 ml. of buffer containing acetylcholine alone. At zero time tubes A and B are thoroughly mixed and 0·5 ml. of the mixture is placed in the side arm of the Warburg vessels using a Krogh-Keys syringe pipette. The contents of the side arm of the first pair (D and U) are tipped into the centre compartment as soon as possible (1 min. cold). The others are placed in the 37° bath and at various times the contents of the side arms are tipped into the (acetylcholine + inhibitor) or acetylcholine alone. Great care should be taken to ensure that the two flasks are treated identically in the time of placing in the bath and in the time of the mixing with acetylcholine. If necessary, due to the mixing times, the flasks were

Table 5. *Reversibility experiments by dilution technique*

(Technique as described on p. 453. *D* and *U* = diluted and undiluted with respect to inhibitor respectively. Error of normal cholinesterase estimation = 0·11 ± 0·056 (11 pairs of estimations).)

Inhibitor			DFP		E 600		E 605		Q 2		Eserine		Nitrogen mustard	
Conc. before dilution (M)			$6·8 \times 10^{-7}$		$4·5 \times 10^{-8}$		$1·7 \times 10^{-6}$		$3·4 \times 10^{-5}$		$7·7 \times 10^{-6}$		$1·6 \times 10^{-4}$	
Time cold (min.)	Time at 37° (min.)	Flask	CO_2 output (μl./min.)	$D-U$	CO_2 output (μl./min.)	$D-U$	CO_2 output (μl./min.)	$D-U$	CO_2 output (μl./min.)	$D-U$	CO_2 output (μl./min.)	$D-U$	CO_2 output (μl./min.)	$D-U$
1	0	*D*	4·64		4·86		5·36		4·96		3·21		4·86	
		U	4·57	0·07	4·76	0·10	5·08	0·28	3·03	1·93	0·85	2·36	4·12	0·74
3	5	*D*	4·33		4·23		4·87		4·26		3·24		4·58	
		U	4·07	0·26	3·98	0·25	4·62	0·25	3·01	1·25	0·82	2·42	4·03	0·55
3	10	*D*	3·43		3·33		4·42		4·25		3·05		3·91	
		U	3·11	0·32	2·98	0·35	4·12	0·30	2·81	1·44	0·72	2·33	3·53	0·38
3	20	*D*	2·60		2·20		3·73		3·89		3·19		3·21	
		U	2·40	0·20	2·12	0·08	3·63	0·10	2·60	1·29	0·77	2·42	2·76	0·45
3	40	*D*	1·67		1·09		2·60		3·53		3·25		2·19	
		U	1·33	0·34	0·89	0·20	2·35	0·25	2·35	1·18	0·72	2·43	1·94	0·25
Activity of red cells before inhibition			4·75 4·92		4·93 5·03		5·60 5·75		4·81 4·86		5·31 5·39		4·65 4·75	
Mean			**0·24**		**0·20**		**0·24**		**1·42**		**2·39**		**0·47**	
Standard deviation			±0·09		±0·10		±0·07		±0·27		±0·04		±0·16	
t			3·02*		1·87		3·61*		10·7*		91·3*		4·90*	
Recalculation using a correction for 2% inhibition in *U* (see below):														
Mean			**0·18**				**0·16**						**0·41**	
Standard deviation			±0·11				±0·07						±0·15	
t			1·35				1·39						4·33	

* Significant $P < 0·05$.

gassed with 5% CO_2 + 95% N_2 after mixing with acetylcholine. In this technique a dilution of ten times was obtained. Before each reversibility experiment a test was carried out to determine the highest concentration of inhibitor which would allow the acetylcholine to stop the reaction at the time of mixing. This was done by adding fresh red-cell suspensions to a mixture of acetylcholine and inhibitor as in the *U* flask described above. In order to be on the safe side, half the highest concentration to give an output of carbon dioxide linear with respect to time under these conditions has been used. In general, it appears that a concentration of inhibitor which does not produce more than 80% inhibition in 40 min. is satisfactory for this purpose. DFP, E 605, E 600 and Q 2 have been examined in this way. An experiment with eserine is included for comparative purposes as a known completely reversible inhibitor and also one with nitrogen mustard which Thompson (1947) has stated is partially reversible (determined by a washing technique on the insoluble portion of brain homogenate).

Examination of the reversibility data given in Table 5 for E 600, E 605 and DFP indicates that a *t* test of the difference between *D* and *U* shows significance when tested against the value of 0·11 ± 0·056 for the ordinary determination of cholinesterase. The table also includes a recalculation of the results assuming that the inhibition in the *U* flask was not quite stopped by the acetylcholine and that 2% inhibition resulted. It can be seen that the results then cease to be significant. It is thought that in a test of this nature the results do not indicate any measurable reversibility. Nitrogen mustard shows some reversibility though it is interesting that the inhibition due to this compound is also progressive. Inhibition by Q 2 is also partly reversible and this observation has been checked by the direct test of inhibiting red cells and then washing with saline. The results in Table 6 confirm this reversibility.

Table 6. *Reversibility of Q 2*

(Cells incubated with $4·8 \times 10^{-5}$ M-Q 2 for 30 min. at 37°)

No. of washings	Inhibition (%)
0	57
1	29
2	27
4	29

Rate of reaction of inhibitors with cholinesterase. It has been shown that the inhibition due to DFP is

a progressive one both with serum cholinesterase (Mackworth & Webb, 1948) and with the cholinesterase of the electric eel (Nachmansohn *et al.* 1948).

The rates of reaction of E 605, E 600 and Q 2 have been determined with cholinesterase of intact red cells using double-armed flasks so that the inhibitor

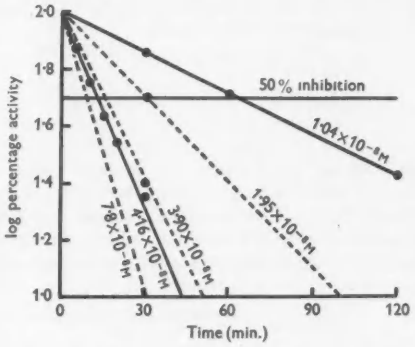


Fig. 1. Rate of inhibition of cholinesterase by E 600. Concentration of E 600 shown against each curve.

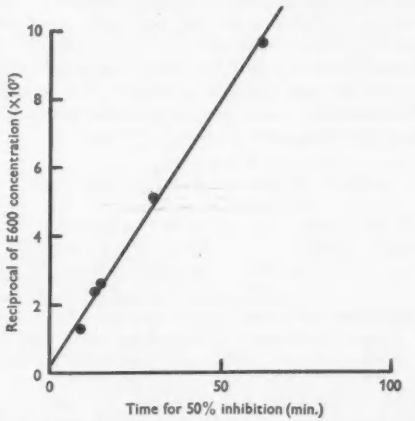


Fig. 2. Demonstration that time for 50% inhibition is inversely proportional to concentration of E 600. Data taken from Fig. 1.

may be added at 37° and the reaction stopped at the desired time by the addition of substrate from the other side arm. The results of these experiments are shown in Figs. 1 and 3 where log percentage activity is plotted against time in minutes. With E 605 and E 600 straight lines are obtained indicating that the reaction shows the characteristics of a unimolecular reaction at a particular concentration of inhibitor.

$$k_1 t = \ln \frac{a}{a-x},$$

where a = original activity, x = decrease in activity due to inhibitor, t = time in min. and k_1 = velocity constant, or

$$k_1 t = \ln \frac{100}{b}, \qquad (1)$$

where b = percentage residual activity.

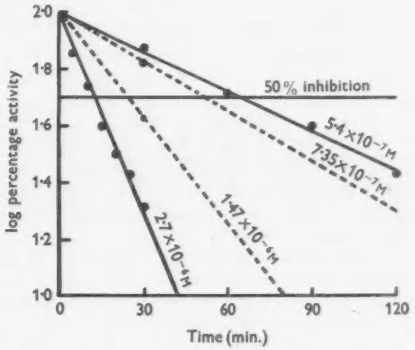


Fig. 3. Rate of inhibition of cholinesterase by E 605. Concentration of E 605 shown against each curve.

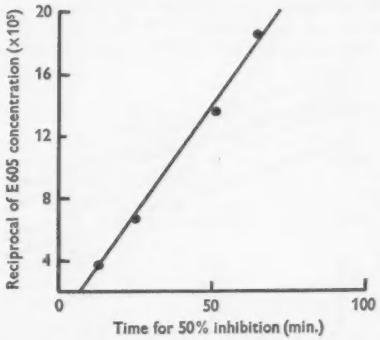


Fig. 4. Demonstration that time for 50% inhibition is inversely proportional to concentration of E 605. Data taken from Fig. 3.

Equation (1) is readily derived from the conventional formula for a bimolecular reaction when one of the reactants is in excess, i.e. in this case

$$K = \frac{1}{tI} \ln \frac{100}{b}, \qquad (2)$$

where I = molar inhibitor concentration. When I is a constant $kI = k_1$ and we have equation (1). Further when $b = 50$ and $t = t_{0.5}$ then

$$t_{0.5} = \frac{1}{kI} \ln 2 = \frac{1}{k_2 I}. \qquad (3)$$

On plotting the times for 50 % inhibition ($t_{0.5}$) against the reciprocal of inhibitor concentration straight lines are obtained, indicating that the reaction, or one of the slowest of a series of reactions, is a bimolecular reaction (cf. Figs. 2 and 4). Using these data, the value of k, the velocity constant, has been calculated, using equation (2).

Table 7. *Velocity constants for bimolecular reaction*

Inhibitor	k (l.mol.$^{-1}$ min.$^{-1}$)
E 600	$1{\cdot}1 \times 10^{6}$
E 605	$2{\cdot}0 \times 10^{4}$

The values for k given in Table 7 are much more satisfactory constants for such irreversible inhibitors than the conventional 50 % inhibition value, e.g. the inhibition at any concentration and time may be readily calculated from k.

When the rate of reaction of Q 2 with cholinesterase is examined on the same basis it is seen that straight lines are again obtained (Fig. 5) on plotting log percentage activity against time, but they differ in two important respects from those of E 600 and E 605. First, the lines produced back do not pass through the origin (2 = log 100 % activity), and secondly the lines for different concentrations are roughly parallel.

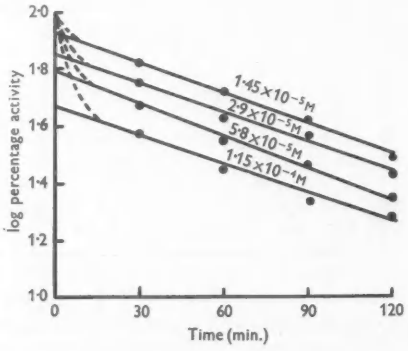


Fig. 5. Rate of inhibition of cholinesterase by Q 2. Concentration of Q 2 shown against each curve.

It has previously been shown that the inhibition of cholinesterase by Q 2 is partly reversible. Possibly in this case the straight lines give a measure of rate of 'becoming irreversibly inhibited', and the amount below the origin where the line produced back-crosses the ordinate is a measure of the reversible inhibition. Reading from the graph on this basis the following figures for reversible inhibition are obtained (Table 8).

Examination of data from the dilution technique, which will give a low result, gives approximately 25 % reversibility for $3{\cdot}4 \times 10^{-5}$ M, while the direct-washing test gave 29 % for $4{\cdot}5 \times 10^{-5}$ M, both in fair agreement with the above.

Table 8. *Percentage inhibition at zero time by Q 2 extrapolated from Fig. 4*

Concentration of Q 2 (M)	Inhibition at zero time (%) (Reversible)
$1{\cdot}45 \times 10^{-5}$	16
$2{\cdot}9 \times 10^{-5}$	29
$5{\cdot}8 \times 10^{-5}$	38
$1{\cdot}15 \times 10^{-4}$	54

The second point, that the straight lines for different concentration of inhibitor are parallel, indicates that under these conditions the rate of the reaction leading to irreversibility (the slowest in the system) is independent of concentration, i.e. as in a unimolecular reaction where

$$t_{0.5} = \frac{\ln 2}{k}.$$

Affinity of inhibitors for cholinesterase. Several workers have shown that the inhibition produced by some agents is prevented if the substrate, acetylcholine, is added first, for example, Thompson (1947) and Barron, Bartlett & Miller (1948) with the nitrogen mustards and Burgen (1949) with TEPP. It is shown here that the inhibition due to E 605, E 600 and DFP is also prevented if the substrate is added first (Table 9).

Table 9. *Protection of cholinesterase from inhibition by substrate*

Inhibitor	Concentration (M)	Inhibition: Inhibitor added before substrate (%)	Inhibition: Inhibitor added after substrate (%)
E 605	$1{\cdot}72 \times 10^{-6}$	60	3
DFP	$6{\cdot}8 \times 10^{-7}$	70	3
E 600	$4{\cdot}5 \times 10^{-8}$	73	4

These results suggest that the inhibitor attaches itself to the group or groups involved in the hydrolysis of acetylcholine.

Mendel *et al.* (1943) have shown that benzoylcholine and acetyl-β-methylcholine may be used as specific substrates for pseudo and true cholinesterase respectively. Table 10 shows that benzoylcholine acts as an inhibitor for true cholinesterase, indicating that although it is not hydrolysed by the 'true' enzyme it is attached to the active centres.

Butyrylcholine has also been shown to be a substrate for pseudo-cholinesterase (Easson & Stedman, 1936; Nachmansohn & Rothenberg, 1945). Cohen, Kalsbeck & Warringa (1949), using a continuous titration method, have reported that butyryl-

choline can also act as a competitive inhibitor for the true cholinesterase. Using the Warburg technique we have obtained the following few results. It seems that butyrylcholine is roughly three times as effective as an inhibitor as benzoylcholine. However,

Table 10. *Inhibition of specific cholinesterase by benzoylcholine*

(Conc. of acetylcholine = $1{\cdot}35 \times 10^{-2}$ M)

Conc. of benzoylcholine (M)	Inhibition (%)
$1{\cdot}24 \times 10^{-1}$	77
$6{\cdot}12 \times 10^{-2}$	71
$2{\cdot}04 \times 10^{-2}$	44
$9{\cdot}62 \times 10^{-3}$	25
$8{\cdot}3 \times 10^{-3}$	24
$5{\cdot}64 \times 10^{-3}$	15
$1{\cdot}92 \times 10^{-4}$	2

From above data 50% inhibition would be obtained with $2{\cdot}52 \times 10^{-2}$ M-benzoylcholine.

Therefore for 50% inhibition $\frac{[\text{Benzoylcholine}]}{[\text{Acetylcholine}]} = \frac{1{\cdot}86}{1}$.

Table 11. *Inhibition of specific cholinesterase by butyrylcholine*

(Conc. of acetylcholine = $1{\cdot}35 \times 10^{-2}$ M)

Conc. of butyrylcholine (M)	Inhibition (%)
$5{\cdot}97 \times 10^{-3}$	41
$5{\cdot}97 \times 10^{-4}$	9
$5{\cdot}97 \times 10^{-5}$	0

50% inhibition at approx. $9{\cdot}1 \times 10^{-3}$ M-butyrylcholine.

At 50% inhibition $\frac{[\text{Butyrylcholine}]}{[\text{Acetylcholine}]} = \frac{0{\cdot}67}{1}$.

since we did not learn of the butyrylcholine inhibition until most of this work had been completed, benzoylcholine has been used for all of the work to be described in this paper. That the inhibition due to benzoylcholine is readily reversible can be shown by the washing technique described earlier with eserine (Table 3).

Table 12. *Reversibility of benzoylcholine inhibition*

Conc. of benzoylcholine (M)	Inhibition: Before centrifuging (%)	Inhibition: After centrifuging and resuspending in fresh buffer (%)
$1{\cdot}21 \times 10^{-2}$	31	1
$4{\cdot}03 \times 10^{-3}$	10	0

Since, as has been shown above, true cholinesterase has an affinity for benzoylcholine, but does not hydrolyse it (at least, not at a measurable rate), it was thought that this property might be utilized for the determination of the affinity of inhibitors for cholinesterase. This cannot be readily done with acetylcholine because the substrate concentration is being continuously depleted. Koelle (1946) has done similar experiments using neostigmine as the reversible and DFP as the irreversible inhibitor. He dialysed to remove neostigmine and DFP after a definite time and measured the inhibition due to DFP. The principle of the method described here has been to incubate a constant concentration of inhibitor and cholinesterase (intact red cells) in the presence of various concentrations of benzoylcholine. After a definite time interval the cells are centrifuged down and resuspended in buffer, thus removing benzoylcholine from the enzyme (Table 12) and also removing excess inhibitor.

In detail the technique is as follows: in a series of centrifuge tubes are placed 5 ml. of a red-cell suspension in buffer (washed with saline three times and diluted to seven times the original volume of whole blood) and 1 ml. of varying concentrations of benzoylcholine in buffer. The solutions are now warmed to 37° and then 1 ml. of inhibitor solution in buffer, also warmed at 37°, is added in sufficient concentration to give 50–75% inhibition. These mixtures are now incubated for 30 min., when they are centrifuged and resuspended in buffer and diluted to their original volume (7 ml.). 3·5 ml. of the contents of each tube are now taken for the determination of cholinesterase activity in the usual way. The value of k, the velocity constant (see above), has been calculated for inhibitor alone and for each concentration of benzoylcholine, and by plotting log k against log M-concentration of benzoylcholine the concentration necessary to reduce k by one-half has been roughly extrapolated.

Table 13. *Affinity of E 600 and E 605 for cholinesterase*

Conc. of benzoylcholine (M)	Activity (%)	k
Inhibitor, E 600 ($4{\cdot}16 \times 10^{-8}$ M)		
$1{\cdot}2 \times 10^{-2}$	84·4	$0{\cdot}14 \times 10^{6}$
$7{\cdot}98 \times 10^{-3}$	77·2	$0{\cdot}21 \times 10^{6}$
$5{\cdot}32 \times 10^{-3}$	70·1	$0{\cdot}29 \times 10^{6}$
$3{\cdot}55 \times 10^{-3}$	60·6	$0{\cdot}39 \times 10^{6}$
0	26·9	$1{\cdot}05 \times 10^{6}$

Concentration to reduce k by one-half = $2{\cdot}1 \times 10^{-3}$ M-benzoylcholine (by extrapolation).

Inhibitor, E 605 ($1{\cdot}17 \times 10^{-6}$ M)		
$1{\cdot}2 \times 10^{-2}$	94·1	$0{\cdot}17 \times 10^{4}$
$4{\cdot}03 \times 10^{-3}$	77·7	$0{\cdot}74 \times 10^{4}$
$1{\cdot}34 \times 10^{-3}$	66·3	$1{\cdot}20 \times 10^{4}$
$4{\cdot}46 \times 10^{-4}$	55·4	$1{\cdot}73 \times 10^{4}$
0	48·3	$2{\cdot}13 \times 10^{4}$

Concentration to reduce k by one-half = $1{\cdot}9 \times 10^{-3}$ M (by interpolation).

It has been seen that the slowest reaction taking place between E 600 (or E 605) and true cholinesterase is a bimolecular one. The mechanism for the slowing down of the reaction by benzoylcholine must be by blocking some of the enzyme centres so that they are no longer available for the inhibitor. Since the concentration of benzoylcholine necessary to

reduce the velocity constant by one-half is the same for both E 600 and E 605 the bimolecular reaction is probably the primary combination of inhibitor with enzyme.

DISCUSSION

No reversibility of E 605, E 600 and DFP can be demonstrated. This has been examined by two methods, one by washing inhibited intact red cells and the other by a modification of the dilution method of Nachmansohn *et al.* (1947). The latter method gives unreliable results since the progressive inhibition characteristic of these inhibitors is not stopped at the time of dilution; during the time between dilution and the addition of acetylcholine, the inhibition of the sample undiluted with respect to inhibitor concentration will be increasing at a far greater rate than the one diluted with buffer alone. By modifying the procedure so that acetylcholine is added at the time of dilution it has been possible to conclude that neither DFP, E 600 nor E 605 exhibit any measurable reversibility *in vitro*.

Examination, on the basis of a homogeneous system, of the data obtained on the rate of inhibition of cholinesterase by E 600 and E 605 indicates that they follow the kinetics of a bimolecular reaction, i.e. the time for 50 % inhibition is inversely proportional to the concentration of the inhibitor. For each particular concentration of inhibitor the reaction shows unimolecular properties, presumably because the concentration of inhibitor is in large excess compared with the concentration of enzyme. The slowest reaction taking place in the system is therefore bimolecular. Velocity constants have been calculated for the reaction with E 600 and E 605; such constants are more satisfactory than the conventional 50 % inhibition value though they are only applicable directly to irreversible inhibitors. Fig. 6 shows a 15 min. inhibition curve for E 605 (percentage inhibition plotted against the negative logarithm of the M-concentration of inhibitor) calculated from the velocity constant

$$(k = 2{\cdot}0 \times 10^4 \text{ l.mol.}^{-1} \text{ min.}^{-1}).$$

This curve shows the usual sigmoid characteristics. Included on the graph are experimentally determined values of inhibition for 15 min. incubation at 37°. Good agreement with theory is shown.

It has been shown that benzoylcholine is a competitive inhibitor of true cholinesterase; utilizing this fact a technique to determine the affinity of these inhibitors for cholinesterase has been developed. This affinity has been expressed as the concentration of benzoylcholine necessary to reduce k, the velocity constant, by one-half. For E 600 and E 605, this concentration is approximately the same; this indicates that the slowest reaction (the bimolecular reaction) is the primary reaction of inhibitor with enzyme active centre.

A study of Q 2 has shown that its inhibition of cholinesterase is partly reversible and the rate of increase of irreversible inhibition is extremely slow. The reversible inhibition shows up when a plot of log percentage activity is made against time. Straight lines are obtained which produced back do not pass through the origin. Reversibility may be demonstrated by this method, which has been used to confirm that E 600 and E 605 are not reversible. An examination of the rate of inhibition with Q 2 shows that it is independent of inhibitor concentration, i.e. unimolecular.

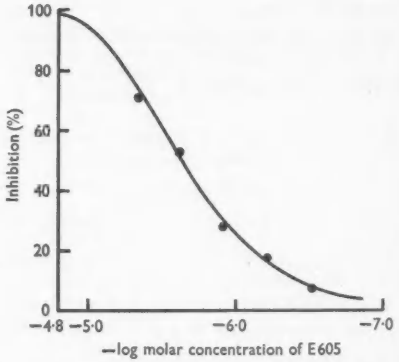


Fig. 6. Theoretical inhibition curve for E 605 calculated from $k = 2{\cdot}0 \times 10^4$ min.$^{-1}$ for incubation time of 15 min. and temperature 37°. Experimentally determined points have been added to show agreement with theory.

It has been shown for several inhibitors that inhibition is prevented by the presence of substrate with the enzyme before the inhibitor (Thompson, 1947; Barron *et al.* 1948; Burgen, 1949). The inhibition by E 600 and E 605 is similarly prevented; the inhibitor and acetylcholine must therefore have some common point of attachment to the enzyme. Since E 600, E 605 and Q 2 are structurally similar molecules, there is no reason to suppose that they will attach themselves to different sites on the enzyme, and any progressive irreversible inhibition will be produced by a similar mechanism for all three compounds. The order of their reaction rates leading to irreversible inhibition is the same as their lability to hydrolysis, i.e. the most stable one, Q 2, is the slowest inhibitor and E 600, the least stable, the most rapid. Hydrolysis of the inhibitors renders them inactive; the substituted phosphoric acid and *p*-nitrophenol and 8-hydroxyquinoline do not inhibit cholinesterase.

A possible mechanism of inhibition by these compounds can now be suggested (see below).

In the case of E 600 and E 605, the bimolecular reaction (1) is the limiting factor. With Q 2 the unimolecular reaction (2) is the slowest. Partial reversibility has been demonstrated, indicating the existence of the reversible inhibitor complex (reaction 1). Since the more stable these compounds are to hydrolysis the slower do they irreversibly inhibit cholinesterase, it is probable that reaction (2) is in fact hydrolysis of the inhibitor at the phosphate bond to liberate from E 600 and E 605, *p*-nitrophenol and from Q 2, 8-hydroxyquinoline. In this case, of course, water would be involved in the reaction making it pseudo-unimolecular. We have attempted to detect *p*-nitrophenol after the inhibition of rat-brain homogenate, the most potent preparation we had at the time, but were unable to get any positive

$$\underset{\text{Enzyme}}{E} + \underset{\text{Inhibitor}}{I} \overset{(1)}{\rightleftharpoons} \underset{\substack{\text{Reversible enzyme}\\ \text{inhibitor complex}}}{(EI)^R} \overset{(2)}{\rightarrow} \underset{\substack{\text{Irreversibly}\\ \text{inhibited enzyme}}}{(E)^I} + \overset{\text{other reaction products}}{\text{---------}}$$

results. Little information is available about the substituted phosphoric acid portion of the molecule. Brauer (1948) claims with HETP containing ^{32}P that no radioactive P remained on the cholinesterase.

From the above mechanism of inhibition it can be deduced that any inhibitor of this type (those producing progressive irreversible inhibition) showing bimolecular characteristics on reaction with cholinesterase, cannot show any reversible inhibition. On the other hand, unimolecular properties should indicate some reversible inhibition.

The concept of a bimolecular reaction may be used to give information about the concentration of enzyme. From Fig. 1 it can be seen that at a concentration of $1{\cdot}0 \times 10^{-8}$ M-E 600 a straight line is obtained. Since straight lines in a graph of this nature are obtained for bimolecular reactions only when one of the reactants is in excess, it may be possible to put an upper limit on the molar concentration of enzyme (active centres). One can easily calculate that when the inhibitor is not more than four times the enzyme concentration the line will be curved more than can be accounted for by experimental error. Therefore it can be said that, since the reaction is bimolecular (1 mol. enzyme active centre to 1 mol. inhibitor), the enzyme concentration which produces 5–6 μl. of CO_2/min. under the conditions described cannot be more than $2{\cdot}5 \times 10^{-9}$ M, and may be much lower. Nachmansohn *et al.* (1948), in their studies on the kinetics of DFP inhibition using electric tissue cholinesterase, state that the usual concentration of cholinesterase in manometric work is 10^{-11} M and that at this concentration 100,000 mol. DFP are required per mol. of cholinesterase to cause 100 % inhibition. This concentration of enzyme was based on a molecular weight of 3,000,000 determined from ultracentrifuge data. The correct result is probably between these values 10^{-11} M and $2{\cdot}5 \times 10^{-9}$ M.

SUMMARY

1. The properties of a convenient preparation of true cholinesterase are described.

2. The available methods for the demonstration of reversibility have been examined. The 'dilution technique' of Nachmansohn has been modified and new methods have been devised. *p*-Nitrophenyl diethyl thiophosphate, *p*-nitrophenyl diethyl phosphate and di-*iso*propyl fluorophosphonate do not show any significant reversibility. 8-Quinolyl diethyl thiophosphate and nitrogen mustard are partly reversible.

3. The rate of progressive inhibition of cholinesterase by *p*-nitrophenyl diethyl phosphate and *p*-nitrophenyl diethyl thiophosphate follows the characteristics of a bimolecular reaction.

4. The rate of progressive inhibition of cholinesterase by 8-quinolyl diethyl thiophosphate shows unimolecular reaction characteristics.

5. Benzoylcholine has been demonstrated to be an inhibitor of specific cholinesterase. A technique has been devised, using this property, to determine the affinity of inhibitors for cholinesterase.

6. A possible mechanism of inhibition based on the above data has been proposed.

7. A maximum figure for the M-concentration of enzyme active centres has been deduced.

Since the completion of the work we have learnt from Mr B. Topley that the sample of E 605 contained some of the isomers *OO*-diethyl-*S*-*p*-nitrophenyl thiolphosphate and *OS*-diethyl-*O*-*p*-nitrophenyl thiolphosphate.

My thanks are due to Mr B. Topley, Messrs Albright and Wilson Ltd., for providing the purified samples of E 600, E 605 and Q 2, to Messrs Boots Pure Drug Co. Ltd. for the sample of nitrogen mustard, to Mr D. R. Davies, Ministry of Supply, Chemical Defence Experimental Establishment, Porton, for the DFP and to Miss J. E. Cremer for valuable technical assistance.

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Studies in Detoxication

32. THE INFLUENCE OF BROMOBENZENE AND CYSTINE ON THE BROMINE CONTENT OF THE HAIR OF RATS

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The ultimate objective of this investigation was to find out how orally administered compounds possessing therapeutic activity could be made to appear in the skin and hair. It was thought that some evidence concerning this could be obtained by feeding compounds which form mercapturic acids *in vivo*, because cystine is much involved in the elaboration of hair and skin and in the formation of mercapturic acids. For this study, bromobenzene was selected as the mercapturic acid former, because it could be traced by its bromine content. It was possible that *p*-bromophenylmercapturic acid might find its way to the skin and hair in quantities sufficient to warrant studying, in a similar manner, mercapturic acid formers carrying therapeutic groups.

It has long been known that arsenic, when administered to animals, appears in the hair, skin and nails, and this is explained by the fact that arsenic compounds combine with sulphydryl groups *in vivo*. Haddow, Elson, Roe, Rudall & Timmis (1945) have shown that when the flavine, 9-phenyl-5:6-benzo-*iso*alloxazine, is injected into albino rats, an orange-yellow pigmentation of the growing parts of the hair appears. The pigment of the hair was shown to be either the original flavine or a simple derivative spectroscopically indistinguishable from it. The results of analyses of the sulphur compounds (inorganic and ethereal sulphates and neutral sulphur) of the urine of the injected animals were consistent with mercapturic acid formation, and it was suggested that the flavine might have been transported to the hair in combination with the cystine used for the elaboration of keratin. Furthermore, Crabtree (1944, 1945, 1946) has shown that a number of mercapturic acid formers, including bromobenzene, when applied locally to the skin, are probably detoxicated by mercapturic acid formation in the skin. At the same time there is a temporary decrease in the glutathione content of the skin and a delay in chemical carcinogenesis caused by 3:4-benzpyrene applied simultaneously. There is therefore ample evidence of a close connexion between the metabolism of mercapturic acid formers and keratin containing tissues.

In the present work, the plan was to feed varying amounts of bromobenzene and L-cystine to different groups of rats which had been clipped free of hair at the beginning of the experiment, and then, after an interval sufficient to allow the new hair to grow to normal length, to analyse the hair and certain organs for bromine and the hair for cystine. It will be shown that in animals showing signs of cystine deficiency as a result of feeding bromobenzene, the bromine content of the hair is much higher than that of other tissues examined.

METHODS

Animals. Twenty-four female albino rats (average weight 110–120 g. and 3–3·5 months old) were divided into six groups, the total weight of rats in each group being approximately equal. Each rat was individually marked, and periodically weighed.

At the beginning of the experiment each rat was clipped (under ether anaesthesia) as closely as possible with electric clippers over the whole of the body excluding the head forward of the ears. After weighing, each group was placed in a wire cage over a large funnel which had been coated with paraffin wax for better drainage of urine. A glass ball, suspended just below the orifice of the funnel, served to separate the faeces from the urine.

On the 30th day after the beginning of the experiment three rats in each group were killed by a blow on the back of the head. The hair was clipped, and the liver, kidneys and muscles of the thigh were removed and stored in 95% ethanol overnight.

One rat in each group was kept for 44 days after the beginning of the experiment, but from the 30th day it was fed on normal stock diet with no added cystine or bromobenzene. On the 44th day it was killed and its hair and tissues analysed for bromine.

Diet. The stock diet consisted of Lever cubes. In our experience this diet is nutritionally sound and rats breed and grow well on it. On analysis these cubes were found to contain approximately 0·5% cystine, 6% fat, 17% protein, and 10% moisture. The bromine content was found to be 34 μg./g. The amount of food eaten each day was determined in a pilot experiment and the rats were thereafter given a definite amount of food each morning. The rats gradually increased their food consumption as they became accustomed to the bromobenzene. At first they ate 10 g. a day and lost weight; later they ate 14 g. a day. The food containers were so arranged that loss of food by spilling was negligible. Water was allowed *ad lib.*

Bromobenzene (b.p. 156°) and L-cystine were administered by mixing with the ground Lever cubes. Cystine was mixed with the food by stirring and shaking. The bromobenzene was at first added to the dry food from a microburette, but it was soon clear from the mercapturic acid output (Table 2) that incomplete mixing enabled the rats to pick out particles of food not contaminated with bromobenzene. Another method of mixing was tried and used. From the sixth day of the experiment, the bromobenzene was measured into a test tube, shaken up into an emulsion with water and then thoroughly mixed with wetted food powder. As a result there was an immediate rise in mercapturic acid output. The amounts of L-cystine and bromobenzene added to the diet of each group is shown in Table 1.

Table 1. *Amounts of* L-*cystine and bromobenzene added to diet of rats*

Group	Cystine added (g./100 g. of diet)	Bromobenzene added (g./100 g. of diet)	Bromobenzene (approximate dose; g./kg. of rat)
I	0·2	0·5	0·6
II	0·4	1·0	1·2
III	0·7	1·5	1·8
IV	1·0	2·0	2·4
V	0	1·5	1·8
VI	0	0	0

Determination of p-*bromophenylmercapturic acid in urine.* The method used was that of Stekol (1936) with minor modifications. The urine of each group of rats was collected each day just before feeding. The sides of each funnel were washed with 25 ml. of 0·05 N-NaOH and the washings added to the urine which was then stored in the refrigerator. Pooled 3-day urines were analysed.

For the determination of mercapturic acid, the urine of each group was centrifuged, and the dark brownish red clear urine was transferred to a 250 ml. measuring flask. The residue in the centrifuged tube was washed twice with 10 ml. of dilute NaOH, centrifuged and the washings added to the main bulk of urine which was diluted to 250 ml. with water.

Table 2. p-*Bromophenylmercapturic acid excretion by rats receiving bromobenzene with and without cystine*

(Dose of bromobenzene: group I, 0·6 g./kg.; groups III and V, 1·8 g./kg. Groups I and III received cystine, group V did not. Figures for groups II and IV are not included in this table (but see Fig. 2). Group VI (controls) excreted no mercapturic acid. Each group contained 4 rats. M.A. = *p*-bromophenylmercapturic acid.)

	Total M.A. excreted in 3 days (g.)			M.A./rat/day (mg.)			Bromobenzene conjugated as M.A. (% of dose)		
Group ...	I	III	V	I	III	V	I	III	V
Period (days)									
1–3	0·115	0·331	0·288	9·6	27·6	24	9·6	9·2	8·0
4–6	0·172	0·403	0·360	14·4	33·6	30	14·4	11·2	10·0
7–9	0·423	1·004	0·879	36	84	73	25·2	19·6	17·2
10–12	0·454	1·307	0·734	38	109	61	26·4	25·6	14·4
13–15	0·505	1·437	0·615	42	120	51	29·2	28·0	12·0
16–18	0·553	1·562	0·611	46	130	51	32·4	30·4	—
19–21	0·564	1·701	0·517	47	142	43	33·2	33·2	—
22–24	0·600	1·639	0·378*	50	137	42	34·8	32·0	—
25–28	0·576	1·599	0·351†	48	133	39	34·0	31·2	—
28–30	0·562	1·557	0·222†	47	130	37	33·8	30·4	—
Total	4·524	12·540	4·755	—	—	—	—	—	—

* Three rats. † Two rats.

Table 3. *Bromine content of the tissues of rats receiving bromobenzene in the diet*

(The stock diet of Lever cubes contained 34 μg. of Br/g.)

	Bromine (μg./g. of tissue)					
	Hair		Muscle		Liver	
Rats killed on ...	30th day	44th day	30th day	44th day	30th day	44th day
Group						
I	149	165	135	71	106	58
II	178	206	122	59	165	69
III	174	214	147	74	151	71
IV	236	295	173	93	168	78
V	605	702	112	54	101	64
VI (controls)	49	47	28	27	35	37

A 50 ml. sample was now treated (to remove interfering substances) with 25 ml. of 20% (w/v) $ZnSO_4$, the mixture vigorously shaken, and 25 ml. of 0·5 N-NaOH added with shaking. The precipitate was removed by filtration, leaving a pale straw-coloured filtrate. For the blank titration 10 ml. of the filtrate, 10 ml. of distilled water, 10 ml. of 95% ethanol, 4 ml. of 2·5 N-HCl and 10 drops of starch solution were mixed and cooled in an ice-salt bath. The solution was titrated with 0·1 N-iodine until a pale blue colour persisted for 30 sec.

For the test titration 10 ml. of the filtrate and 4 ml. of 2·5 N-NaOH was heated on a sand bath for 30 min. The mixture was cooled and finally placed in an ice-salt bath. Water (10 ml.), ethanol (10 ml.), 2·5 N-HCl (10 ml.) and 10 drops of starch were added and the mixture was titrated with 0·01 N-iodine while still in the ice-salt bath. The weight of mercapturic acid in a 3-day urine (in mg.) = (test titre − blank titre) × 3·18 × 50 (318 being the molecular weight of *p*-bromophenylmercapturic acid). The reagents were standardized and recoveries from urine checked against pure *p*-bromophenylmercapturic acid (m.p. 152°) prepared from rat urine according to McGuinn & Sherwin (1933). Recoveries of this acid added to water, normal rat urine and the urine of rats receiving bromobenzene ranged from 96 to 102%.

The mercapturic acid outputs for the five groups are given in Fig. 2 and a complete set of figures for groups I, III and V is given in Table 2.

Distribution of bromine

Hair. For analysis, the hair was well washed with water, then extracted with 95% ethanol for 4 hr. followed by ether for 4 hr. and finally dried *in vacuo* and weighed. The weights of the hair at the beginning and end of the experiment are given in Table 4.

Liver and muscles. These tissues, after keeping overnight in 95% ethanol, were homogenized with acetone in the Waring blender, centrifuged and the acetone layer poured off. The residue was dried at 110° for 6 hr. and kept in stoppered bottles until analysed.

Analysis. Bromine in the hair and tissues was determined by the method of Winneck & Smith (1937*a*) with slight modification. This method entails alkaline ashing of the materials followed by conversion of bromide to bromate which is estimated by iodine-thiosulphate titration. Between 0·1 and 1·0 g. of material was ashed with 1 ml. each of 5 N-KOH, 5 N-K_2CO_3 and 20% sucrose solution. To ensure complete ashing a temperature of 600° was used. The results are given in Table 3.

Cystine content of the hair

The hair was analysed for cystine by hydrolysing for 8 hr. with HCl and formic acid (Miller & du Vigneaud, 1939) and subsequently determining the cystine in the hydrolysate by the method of Folin & Marenzi (1929).

The hair of group V (Table 5) contained 8% less cystine (i.e. 12 instead of 13%) than other groups, and from the work of Lightbody & Lewis (1929) these animals therefore exhibit a cystine insufficiency. Under the microscope, the hair of group V showed a smaller proportion of cortex than the controls, further evidence of cystine deficiency (Smuts, Mitchell & Hamilton, 1932).

Table 4. *Weight of cleaned and dried hair of rats obtained at the beginning and end of the experiment*

(Values for the beginning of the experiment and on the thirtieth day are means for four and three rats respectively; values on the forty-fourth day are for one rat only.)

	Weight of hair/rat (g.)		
Group	At beginning	On 30th day	On 44th day
I	1·963	1·517	1·75
II	1·839	1·603	1·87
III	2·087	1·399	2·13
IV	2·366	1·659	2·01
V	1·909	1·327	1·91
VI	2·256	1·724	2·29
Average	**2·07**	**1·54**	**1·99**

Table 5. *Cystine content of the hair of rats receiving varying amounts of* L-*cystine and bromobenzene*

	Percentage cystine in hair of rats killed on	
Group	30th day	44th day
I (cystine + PhBr)	13·13	13·16
II (cystine + PhBr)	13·18	13·12
III (cystine + PhBr)	13·25	13·29
IV (cystine + PhBr)	13·28	13·24
V (PhBr only)	12·29	12·02
VI (controls)	13·16	13·20

As regards the weight of the hair, it is clear from Table 4 that none of the groups had recovered their full weight of hair in 30 days although the recovery was about 75%. However, if the results for the single remaining rat in each

group are acceptable, then the full weight of the hair has been recovered sometime between the thirtieth and forty-fourth day. The low figure for group V on the thirtieth day is due to the fact that it is made up of hair from three rats, that lived for different periods, i.e. 21, 27 and 30 days, and is probably of no significance.

RESULTS

The growth curves of the rats during the first 30 days of the experiment are shown in Fig. 1. All groups show a loss of weight during the first 12 days, although the loss in the control group VI was slight and ceased after 6 days. The loss in weight initially showed that 10 g. of food/rat/day was not enough, although at first the rats would not eat more if it

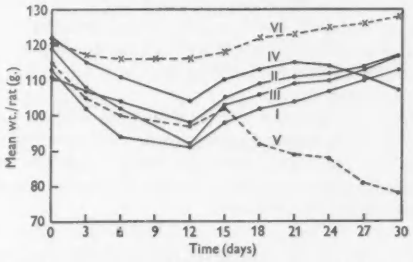

Fig. 1. Growth curves of rats receiving added bromobenzene (PhBr) and L-cystine in their diets (four rats in each group). The amounts of cystine and PhBr are expressed as g./100 g. of basal diet.

I, basal diet + 0·2 g. of cystine + 0·5 g. of PhBr;
II, basal diet + 0·4 g. of cystine + 1·0 g. of PhBr;
III, basal diet + 0·7 g. of cystine + 1·5 g. of PhBr;
IV, basal diet + 1·0 g. of cystine + 2·0 g. of PhBr;
V, basal diet, no cystine + 1·5 g. of PhBr;
VI, controls, basal diet alone.

contained bromobenzene, and on the seventh day they were given 14 g./day each. If the growth curves of groups I–V are compared with that of group VI which had also been clipped, but had a palatable diet from the beginning, then it is clear that the initial loss of weight is largely due to the lower intake of food. As mentioned earlier, after the 6th day the diet was mixed in another way and the rats ate more, and after the twelfth day all groups increased in weight. In groups I–III, this weight increase continued to the end of the experiment. In group IV the increase continued until the twenty-first day and then there was a gradual drop in weight. In group V, however, there was a slight increase for 3 days and then a pronounced loss in weight accompanied by the death of two of the animals in this group, the one on the twenty-first and the other on the twenty-seventh day of the experiment.

The observed changes in weights of the animal can be correlated with the output of mercapturic acid which is shown graphically in Fig. 2. During the first 6 days the percentage of the conjugate was low in all groups, and this corresponded with the period of loss of weight shown in Fig. 1. As the intake of food was low, the intake of bromobenzene was also low and, as suggested earlier, the incomplete mixing of the bromobenzene with the food allowed the rats to select particles of food free of bromobenzene. After the sixth day the output of mercapturic acid rose following better mixing of the bromobenzene with the diet. In groups I, II and III, the output of mercapturic acid tended to reach a steady level and a constant percentage of the dose was excreted as the conjugate. In the case of group IV, the constant percentage conjugation was reached at about the eighteenth to twentieth day, but thereafter there was a drop in the output which corresponded to the drop

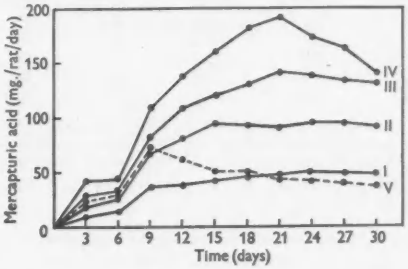

Fig. 2. Excretion of *p*-bromophenylmercapturic acid by rats receiving bromobenzene and L-cystine in varying amounts. For curves I–V, diets are as given in Table 1 and Fig. 1.

in weight of the animals as indicated in Fig. 1. The animals in group IV ate all their food until the twenty-eighth day; from the twenty-eighth to thirtieth day they left some of their food. With group V, there was the initial lag to the sixth day, followed by an increase of short duration and then a gradual fall in output after the ninth day. Here again the changes followed closely the growth curve in Fig. 1. The curve for group V should be compared with that for group III, for both these groups were receiving the same dose of bromobenzene, but the latter group received extra cystine as well. The percentages of bromobenzene excreted as mercapturic acid during the experiment are quoted in Table 6. According to Stekol (1943) the inhibition of growth of rats by bromobenzene is due to loss of cysteine as mercapturic acid. This lack of growth, however, can be prevented by addition of L-cystine to the diet. In the present experiments it appears that groups I–IV have sufficient cystine in their diets to cover the needs of growth, hair elaboration and the formation of mercapturic acid, because they gain weight (after the initial loss already explained), the cystine content

of their hair is normal, and the percentage of bromobenzene conjugated with cysteine is relatively constant. In group IV, however, there is a loss of weight and a drop in mercapturic acid production during the last 6 days of the experiment. This may be due to gradual poisoning by the very large dose of bromobenzene administered, or by the mercapturic acid, for Coombs & Hele (1927) have indicated that, in dogs, *p*-bromophenylmercapturic acid is much more toxic than bromobenzene. Group V seems definitely

Table 6. *Percentage of the dose of bromobenzene excreted as mercapturic acid*

	Percentage of dose excreted as mercapturic acid			
Group	Days 1–6	Days 7–12	Days 12–30	Average for the whole period
I	12	25·9	33	**27**
II	11	25·9	33	**28**
III	10·2	22·6	31	**26**
IV	10·6	21·6	31	**25**
V	9·0	15·8	12*	**13**†

* This figure is for twelfth to eighteenth days, because after the eighteenth day, all the food given was not eaten and therefore the amount of PhBr ingested could not be assessed.

† For 1–18 days.

to suffer from cystine deficiency as indicated by loss in weight; the cystine content of the hair is lower than in other groups and, although this group received the same amount of bromobenzene as group III, after the ninth day excretion of mercapturic acid was less than half the amount excreted by group III.

The distribution of bromine in the tissues examined (Table 3) showed that when bromobenzene is being fed there is an increase in the bromine content of the hair, muscle and liver. It is known that the bromine content of animal tissues varies with the concentration of the bromine of the diet (Winneck & Smith, 1937*b*). In groups I, II and III, where there is no apparent cystine deficiency, the bromine contents of the three tissues examined on the thirtieth day are of the same order, that of hair being slightly higher than liver and muscle. In group IV, the bromine content of the hair is appreciably higher than that of the first three groups, whereas in group V the hair bromine is nearly four times that of other groups. In all groups the bromine contents of the muscle and liver are of the same order. These figures suggest that where there is a cystine insufficiency brought about by bromobenzene feeding, the bromine appears in the hair in enhanced amounts. This further suggests that much of the available cystine in group V and some of it in group IV is being used for conjugation with bromobenzene and that perhaps some *S*-*p*-bromophenylcysteine is being used for elaboration of hair owing to shortage of cystine. The shortage of cystine is apparent in the analyses of hair given in Table 5, which shows that the hair of group V contains less cystine than that of other groups. If it is assumed that the bromine of the hair is present as *S*-*p*-bromophenylcysteine, $BrC_6H_4SCH_2CH(NH_2)COOH$, then it can be calculated that the amount of this compound appearing in the hair of each group on the thirtieth day is I, 33; II, 40; III, 38; IV, 52; and V, 133 mg./100 g. of dry defatted hair. In group V this would amount to about 1% of the cystine present. However, the presence of *S*-*p*-bromophenylcysteine in the hair of these animals has not yet been proved. When bromobenzene is withdrawn from the diet as in the case of the surviving rat in each group which was kept until the forty-fourth day, the bromine content of the liver and muscles tends to return to the normal values obtained on the control group VI. In the case of the hair, however, the bromine content still rises in all groups. This is presumably due to residual bromine in the root which has grown out by the forty-fourth day.

The conclusion which can be drawn from these experiments is that in order to drive bromobenzene into the hair, in quantities which might be considered as 'therapeutic', a state of cystine insufficiency must be produced.

SUMMARY

1. A study has been made of the influence of varying amounts of bromobenzene and L-cystine added to the diet, on the growth, the bromine content of the hair muscle and liver, and the cystine content of the hair of young rats.

2. When the cystine content of the diet was adequate, the animals, when given bromobenzene, excreted a fairly constant amount of mercapturic acid each day, the cystine content of the hair was normal and there was no final loss in weight.

3. When the cystine of the diet was inadequate, bromobenzene feeding caused the animals to lose weight and to excrete less mercapturic acid than animals with adequate cystine. The cystine content of the hair was lower than normal.

4. On feeding bromobenzene with adequate cystine intakes, the bromine contents of the hair, muscle and liver rise above normal to approximately the same extent. On inadequate cystine intakes, the bromine content of the hair is much higher than that of the liver and muscles.

5. The suggestion is made that when bromobenzene is fed to rats on diets containing insufficient cystine for both growth and detoxication, some bromophenylcysteine is incorporated in the hair in an attempt to make good the lack of cystine.

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The Intermediary Metabolism of the Mammary Gland

3. ACETATE METABOLISM OF LACTATING MAMMARY GLAND SLICES WITH SPECIAL REFERENCE TO MILK FAT SYNTHESIS

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The origin of milk fat has been studied by various techniques (see review by Folley, 1949), the most favoured hitherto being the arterio-venous (A.V.) method which, however, can give little insight into the synthetic mechanisms involved. Arterio-venous studies have disproved the earlier idea of the utilization of blood phospholipin fatty acids for milk fat synthesis and have provided evidence that fatty acids of neutral fat, probably belonging to the glyceride fraction, are absorbed from the blood by the lactating udder, and are there transformed into glycerides of milk fat (see reviews by Folley, 1940, 1949).

Because they obtained a high respiratory quotient (R.Q.) by the A.V. method for the lactating goat udder, Graham, Houchin, Peterson & Turner (1938) postulated the formation of fat from carbohydrate by the lactating mammary gland (see also Reineke, Stonecipher & Turner, 1941). In apparent agreement with this finding, which had meanwhile been confirmed for the cow by other workers (see Folley, 1949), were the *in vitro* results of Folley & French (1948*a*, *b*, 1949*c*), who showed that lactating mammary slices from non-ruminants (mouse, rat, rabbit and guinea pig) utilize glucose *in vitro* with R.Q. > 1, results which were also interpreted as indicating the synthesis of fat from carbohydrate. The situation was, however, complicated by the fact that mammary gland slices from ruminants (cow and goat) were found to be practically inert towards glucose *in vitro*. As far as ruminants are concerned, therefore, these slice experiments provided no evidence to support *in vivo* indications of fat synthesis from carbohydrate, and threw little light on the nature of the precursors of milk fat.

Tracer studies have provided evidence of the utilization of acetate for fatty-acid synthesis in the mammal both *in vivo* (Rittenberg & Bloch, 1945) and in liver slices *in vitro* (Bloch & Kramer, 1948). Moreover, it is now known that in ruminants large quantities of acetic acid are produced in the rumen by fermentation of cellulose (Elsden & Phillipson, 1948) and that this acetate can be absorbed, leading to appreciable arterial blood levels (Reid, 1950, for sheep; McClymont, 1949, for cow). The possibility, arising mainly from the first of these tracer studies, that acetate is utilized by the mammary gland for milk fat synthesis was suggested by Folley (1945).

This paper deals with the acetate metabolism of mammary gland slices from various species, and presents evidence for the utilization of acetate for milk-fat synthesis by the lactating mammary gland. Part of this work has already been reported in preliminary communications (Folley & French, 1948*c*, 1949*a*, *b*) in the first two of which attention was drawn to the significance of these results for the origin of the short-chain fatty acids, which, as shown principally by Hilditch and his co-workers (Hilditch, 1947), are a prominent feature of the milk fat of herbivorous animals (although absent from body fat), and the origin of which has been the subject of controversy (see discussion by Folley, 1949). If acetate is utilized for milk-fat formation in the mammary gland, it seems probable that the short-

chain acids are intermediates in the formation of higher fatty acids by condensation of acetate or some 2-carbon derivative thereof (Folley, 1945).

More recently this theory of the origin of the short-chain acids of milk fat has been confirmed by tracer experiments. Popják & Beeckmans (1949*b*) have demonstrated the incorporation of injected acetate labelled with ^{14}C into the glyceride fatty acids isolated from the mammae of 28-day pregnant rabbits. Fractions representing the short-chain acids have subsequently been isolated from these fatty-acid samples and shown to contain higher proportions of isotopic carbon than the non-volatile residues (Popják, Folley & French, 1949). These results leave very little room for doubt that short-chain fatty acids of milk fat can be formed in the mammary gland by condensation of acetate or of a reactive derivative thereof, and in this respect are in harmony with the conclusions drawn from the results presented in this paper.

EXPERIMENTAL

Manometric methods. Quotients (Q_{O_2}, and $Q^{O_2}_{acid}$), calculated in μl./mg. final dry weight/hr., and R.Q. were determined by the method of Dickens & Šimer (1931) which has the advantage that the value for $Q^{O_2}_{acid}$ gives an estimate of the utilization of a single consumable anion, such as acetate, if it is used as substrate. The values of $Q^{O_2}_{acid}$, used throughout this work as a measure of the acetate utilization, probably somewhat underestimate the latter, since this quotient is a resultant of acid production and utilization by the slice, and we have found that mammary gland slices invariably produce some acid when incubated with carbohydrate or without substrate (Folley & French, 1949*c*). Nevertheless, this uncertainty clearly does not affect the conclusions drawn from the results.

The general manometric technique was that used before (Folley & French, 1949*c*), the gas phase being 5% CO_2 in O_2, temperature 37°, and the medium the saline-bicarbonate of Krebs & Henseleit (1932). Most determinations were done in duplicate and all lasted for 3 hr. Na acetate was used in 0·02M final concentration throughout. The acetate solutions were prepared by saturating an aqueous 0·2M solution with the gas mixture at 37°, then adjusting to pH 7·4 (glass electrode) with solid Na_2CO_3. This solution (1 vol.) was added to Krebs saline-bicarbonate (9 vol.).

Animals. Abdominal mammary tissue was obtained and sliced as before (Folley & French, 1949*c*) from hooded Norway rats killed at the fourteenth to twenty-first days of lactation, and from Dutch rabbits killed at various stages. Samples of udder tissue were obtained from lactating 'scrub' goats, whose stages of lactation were unknown, but were probably 8–9 months. The goats were milked twice daily up to the time of slaughter when they were shot and the udder tissue removed immediately. In three cases udder tissue was obtained from living goats under cyclopropane anaesthesia. Udder tissue samples were removed, under cyclopropane anaesthesia, at various known times after parturition from sheep which were suckling lambs, and a final sample taken from each at slaughter under the same conditions as cows (see below). All the sheep samples were obtained in early lactation. Special arrangements were made for cows of known history to be slaughtered in milk. All were of necessity in late lactation (Folley & French, 1949*c*), but wherever possible animals giving at least 10 lb. (4·5 kg.) of milk daily were selected. They were milked twice daily up to the morning of slaughter. At death the udder was at once removed and slices of alveolar tissue cut at the abattoir and brought to the laboratory in oxygenated saline-bicarbonate at room temperature. The extra delay from animal to manometric flask was of the order of 10 min.

RESULTS

Non-utilization of glucose by lactating mammary gland slices from ruminants

We have previously reported (Folley & French, 1949*c*) that lactating mammary gland slices from the goat and cow hardly utilize glucose *in vitro* as shown by the low Q_{O_2} and the fact that the R.Q. is below

Table 1. *Respiration of lactating mammary gland slices from ruminants in presence of glucose* (0·3%)

Animal	Stage of lactation	No. of animals	No. of determinations	$-Q_{O_2}$ Mean*	$-Q_{O_2}$ Range	Q_{acid} Mean	Q_{acid} Range	R.Q. Mean	R.Q. Range
Sheep	7–47 days	4	15†	3·9±0·3	2·0–5·2	1·9±0·2	0·7–2·9	0·88±0·01	0·69–1·05
Goat	Advanced lactation: stages unknown	11	11‡	4·9±0·3	3·2–6·2	1·6±0·1	1·0–2·5	0·86±0·03	0·69–0·95
Cow	Advanced lactation: includes 3 cows giving no more than 5 lb. (2·3 kg.) milk/day at slaughter	9	9	2·7±0·4	1·5–4·7	1·9±0·2	1·0–2·8	0·84±0·03	0·64–0·93
Cow	Advanced lactation: milk yield at slaughter approximately 10 lb. (4·5 kg.)/day	6	6	3·1±0·5	2·0–4·7	1·9±0·2	1·0–2·3	0·84±0·04	0·64–0·93

* In this and subsequent tables results are shown as mean ± S.E.M.
† Includes twelve determinations on biopsy material.
‡ Includes three determinations on biopsy material.

unity, though of course glucose may be utilized for lactose formation. Table 1 includes these and additional data for the goat and cow, together with entirely new data for sheep. Of the latter fifteen determinations, eleven were on biopsy material obtained from sheep under cyclopropane anaesthesia, but these, and also the determinations in presence of acetate (see below), fit very well with the terminal values on tissue obtained at slaughter, so there is no evidence that cyclopropane affected the metabolism of mammary tissue taken at biopsy.

Table 1 shows clearly that ruminant mammary tissue oxidizes very little glucose *in vitro* and that the R.Q. is low. The possibility that the cow and goat tissue exhibited this behaviour because the tissue was from animals in late lactation is disposed of by the results on sheep all of which were studied in early lactation. This conclusion is supported by the fact that if one eliminates those three of the nine cows which gave 5 lb. (2·3 kg.) milk/day or less at slaughter, leaving six giving 10 lb. (4·5 kg.)/day and which are known to have been regularly milked up to the morning of slaughter, the mean R.Q. is no higher.

Utilization of acetate by lactating mammary gland slices

Table 2 gives results for tissue from ruminant and from two non-ruminant animals, rat and rabbit. With ruminant tissue $-Q_{O_2}$ was greater than in the presence of glucose. There was appreciable acetate utilization as shown by negative values of Q_{acid}, and the mean R.Q. for each species was greater than unity. The mean R.Q. values for the goat and cow were shown by the 'one-tail' 't' test to be significantly greater than unity (Table 2). In the case of sheep, since on three of the four animals several results were obtained at different stages of lactation, the standard errors of the means were estimated from the deviations of the weighted squares of 'animal means'. In this case also the observed mean R.Q. was significantly greater than unity. It may be concluded that ruminant mammary tissue actively utilizes acetate *in vitro* with high R.Q. Acetate has previously been shown to be oxidized by kidney cortex of rat and rabbit (Elliott, Benoy & Baker, 1935) and guinea pig (Kleinzeller, 1943), but with R.Q. < 1 (Elliott *et al.* 1935).

By contrast, the results in Table 2 show that tissue from the two non-ruminant forms studied gives a low *in vitro* R.Q. with acetate. Rat tissue showed little or no acetate utilization, the mean $-Q_{O_2}$ and R.Q. values for ten determinations being hardly different from the values for the endogenous respiration of mammary slices from fully lactating rats previously reported (Folley & French, 1949*c*). The values for Q_{acid} in acetate are indeed somewhat lower, 0·1 as against 0·6, but it is doubtful if this difference is sufficiently significant to allow the conclusion that

Table 2. *Respiration of lactating mammary gland slices in presence of acetate (0·02 M)*

Animal	Stage of lactation	No. of animals	No. of determinations	$-Q_{O_2}$ Mean	$-Q_{O_2}$ Range	Q_{acid} Mean	Q_{acid} Range	R.Q. Mean	R.Q. Range	P*
Sheep	7–47 days	4	15†	6·1 ± 0·4	3·1–9·3	− 4·1 ± 0·5	(− 0·4)–(− 7·5)	1·17 ± 0·04	0·93–1·44	0·017
Goat	Advanced lactation: stages unknown	7	7‡	6·1 ± 0·4	5·2–8·4	− 3·9 ± 0·7	(− 2·0)–(− 6·2)	1·17 ± 0·07	0·99–1·46	0·025
Cow	Advanced lactation: includes 3 cows giving no more than 5 lb. (2·3 kg.) milk/day at slaughter	9	9	3·9 ± 0·5	1·7–7·3	− 1·6 ± 0·4	(− 0·1)–(− 4·4)	1·08 ± 0·04	0·89–1·28	0·035
Cow	Advanced lactation: milk yield at slaughter approximately 10 lb. (4·5 kg.)/day	6	6	4·4 ± 0·6	2·8–7·3	− 2·1 ± 0·5	(− 0·8)–(− 4·4)	1·12 ± 0·04	1·01–1·28	0·015
Rat	14–20 days	10	10	5·3 ± 0·3	3·9–7·3	+ 0·2 ± 0·3	(− 0·9)–(1·2)	0·82 ± 0·04	0·60–0·94	—
Rabbit	2–5 weeks	9	9	6·8 ± 0·4	4·9–9·0	− 2·5 ± 0·4	(− 1·0)–(− 4·4)	0·92 ± 0·05	0·74–1·18	—

* On the assumption that the true mean R.Q. is unity, P denotes the probability of obtaining by random sampling a result as large as or larger than the one obtained.
† Includes twelve determinations on biopsy material.
‡ Includes one determination on biopsy material.

a small amount of acetate is utilized by rat tissue. Rabbit tissue seems to occupy an intermediate position between the rat and the ruminants in that though the mean R.Q. was less than unity, $-Q_{O_2}$ was as great as in previously reported experiments with glucose as substrate (Folley & French, 1949*c*), and there was an appreciable uptake of acetate.

Previous studies on rat mammary tissue (Folley & French, 1949*d*) have shown a progressive increase in $-Q_{O_2}$ and R.Q. (in glucose) with advance in lactation (i.e. as the rate of milk secretion increases), at least in the earlier stages. It was therefore of interest to find out if the same applies to ruminant tissue. Fig. 1 shows the relationship between stage of lactation and the quotient values in acetate for tissue from sheep no. 1, a pedigree ewe in excellent condition which was lactating well, as indicated by the growth rate of her single lamb. The period studied was from 7 to 47 days post-partum, which is before the declining phase of lactation sets in (Barnicoat, Logan & Grant, 1949). As lactation progressed there was a progressive increase in $-Q_{O_2}$ and acetate utilization ($-Q_{acid}$) and the R.Q. increased from a value very near unity at 7 days post-partum to a value of almost 1·3 at 47 days. Incidentally these curves illustrate the concordance between values obtained with biopsy specimens and those for tissue obtained at slaughter.

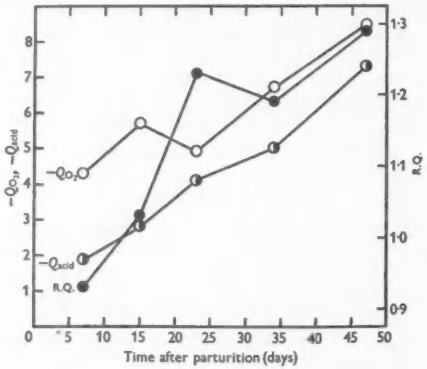

Fig. 1. Changes in respiration and acetate utilization of lactating mammary gland slices from sheep no. 1 with advance of lactation. Slices incubated at 37° in saline-bicarbonate at pH 7·4 with 0·02M-sodium acetate as substrate.

Fig. 2 shows well the contrast between the progressive increase in $-Q_{O_2}$ and R.Q. in acetate as the early phase of lactation progresses, and the constancy or slow fall in these quotients with glucose as substrate. It will be noted that both substrates gave substantially the same values at the outset (7 days).

The remaining three sheep were non-pedigree animals of unknown history, purchased from a dealer. They were in much poorer condition and they lambed down late in the season. All the available evidence indicated that these were not such good milkers as sheep no. 1, and it is not therefore surprising that the results on tissue obtained from them showed no regular correlation with advance of lactation.

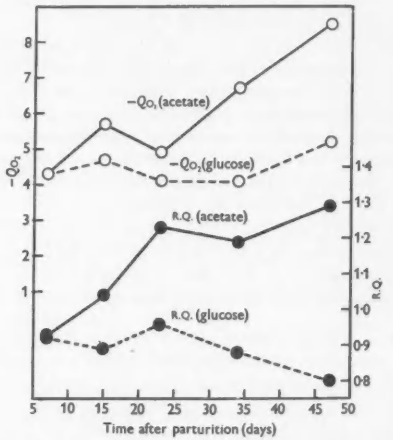

Fig. 2. Comparison of the effect of advancing lactation on the respiration of lactating mammary gland slices from sheep no. 1 in presence of 0·02M-sodium acetate and 0·3% glucose respectively. Slices incubated at 37° in saline-bicarbonate at pH 7·4.

Effect of glucose on utilization of acetate by lactating mammary gland slices

In contrast to tissue from ruminants the failure of mammary gland slices from non-ruminants to utilize acetate with high R.Q. is very intriguing. However, there are indications that rabbit mammary tissue, at least, utilizes some acetate, perhaps partly for fatty-acid synthesis, though if this is so the rate is insufficient to give a net synthesis of fat, assuming that the high R.Q. implies this. It should, therefore, be possible to find conditions under which the rate of acetate incorporation into fat by non-ruminant mammary tissue could be increased.

Bloch & Kramer (1948) found that glucose or pyruvate increased the rate of incorporation of acetate into fatty acids by rat liver slices *in vitro*. We have therefore studied the effect of glucose on the utilization of acetate by lactating mammary slices from rat, rabbit and sheep. In combination with acetate (0·02M) various glucose concentrations were tried, but with rat and rabbit tissue, which readily utilizes glucose, a suboptimal glucose concentration (0·1%) was used in most experiments. In sheep,

a glucose concentration of 0·15 % was used throughout this series of experiments. We have not studied the effect of pyruvate on acetate utilization because our technique can only be used for studying the utilization of a single anion.

Detailed results of experiments allowing direct comparison of results with combined and single substrates on tissue from the same animal, thus leading to Δ values for quotients, are given for rat, rabbit and sheep in Tables 3, 4 and 5 respectively. For rat and rabbit tissues, which utilize glucose but not acetate, the Δ values are (glucose + acetate) − (glucose); for sheep tissue they represent (acetate + glucose) − (acetate). Table 6 gives summarized mean results for series of determinations involving each substrate alone and both combined. In this case parallel determinations on tissue from the same animal are not necessarily involved.

Rat. Mean $-Q_{O_2}$ was slightly greater in presence of both substrates than with glucose alone (Table 6); it was slightly increased in four experiments, hardly changed in one and decreased in one (Table 3). Presence of acetate changed Q_{acid} values from small positive values to definite negative values in all experiments (Table 3), indicating an appreciable uptake of acetate in presence of glucose (Table 6). The changes in R.Q. were slight, but there was no evidence that the utilization of acetate lowered the R.Q. from the high value characteristic of glucose utilization, towards the theoretical value of unity for acetate oxidation.

Rabbit. Mean $-Q_{O_2}$ was appreciably increased by the combined substrates over the value for either substrate alone (Table 6); this effect was observed in nine of the ten experiments recorded in Table 4. It will be noticed that for rabbit tissue the mean $-Q_{O_2}$ was higher in 0·02 M-acetate than in 0·1 % glucose and in fact equal to the value for 0·3 % glucose previously reported (Folley & French, 1949*c*).

Q_{acid} changed from an appreciable positive value in glucose to a relatively large negative value, twice as great as in acetate alone (Table 6); this large acetate uptake in presence of glucose occurred in all experiments in Table 4. These results indicate a considerable utilization of acetate by rabbit mammary tissue when glucose is present. The mean R.Q. for the combined substrates was hardly different from that for glucose, though of course higher than the value for acetate (Table 6); the Δ values were positive in five of the ten experiments recorded in Table 4, and negative in five. Here again there was no evidence that any appreciable proportion of the extra acetate was oxidized by the slices.

Table 3. *Effect of glucose on utilization of acetate by lactating mammary gland slices from rats*

(Acetate always 0·02 M; glucose as shown in column 2. Rats 14–21 days in lactation.)

Rat no.	Glucose added (%)	Glucose			Glucose + acetate			(Glucose + acetate) − (glucose)		
		$-Q_{O_2}$	Q_{acid}	R.Q.	$-Q_{O_2}$	Q_{acid}	R.Q.	$\Delta -Q_{O_2}$	ΔQ_{acid}	Δ R.Q.
21	0·05	9·6	0·3	0·97	8·6	−1·8	1·02	−1·0	−2·1	0·05
21	0·1	9·1	0·2	1·22	10·7	−2·6	1·15	1·6	−2·8	−0·07
21	0·15	9·9	0·6	1·38	11·0	−2·7	1·32	1·1	−3·3	−0·06
22	0·1	8·4	0·4	1·49	9·7	−2·9	1·39	1·3	−3·3	−0·10
23	0·1	7·6	0·5	1·39	8·2	−1·9	1·26	0·6	−2·4	−0·13
24	0·1	7·6	0·7	1·30	10·8	−1·7	1·24	3·2	−2·4	−0·06
Mean of four determinations involving 0·1 % glucose								**1·7**	**−2·7**	**−0·09**

Table 4. *Effect of glucose on utilization of acetate by lactating mammary gland slices from rabbits*

(Acetate always 0·02 M; rabbits 2–5 weeks in lactation.)

Rabbit no.	Glucose added (%)	Glucose			Glucose + acetate			(Glucose + acetate) − (glucose)		
		$-Q_{O_2}$	Q_{acid}	R.Q.	$-Q_{O_2}$	Q_{acid}	R.Q.	$\Delta -Q_{O_2}$	ΔQ_{acid}	Δ R.Q.
98	0·05	—	—	—	5·5	−2·2	1·15	—	—	—
98	0·1	4·0	1·5	1·04	8·8	−4·3	1·30	4·8	−5·8	0·26
98	0·15	6·7	1·1	1·29	8·3	−5·6	1·70	1·6	−6·7	0·41
94	0·15	5·4	0·7	1·43	5·2	−3·0	1·68	−0·2	−3·7	0·25
101	0·1	5·8	1·8	1·28	10·6	−6·3	1·20	4·8	−8·1	−0·08
C4	0·1	5·6	1·1	1·31	10·1	−6·0	1·28	4·5	−7·1	−0·03
C5	0·1	4·2	0·6	1·49	8·3	−4·4	1·23	4·1	−5·0	−0·26
89	0·1	7·5	1·1	1·24	9·8	−4·5	1·46	2·3	−5·6	0·22
108	0·1	6·6	1·0	1·34	11·2	−5·8	1·21	4·6	−6·8	−0·13
114	0·1	4·8	1·0	1·38	8·2	−4·5	1·17	3·4	−5·5	−0·21
70	0·1	5·0	1·4	1·28	8·7	−4·0	1·35	3·7	−5·4	0·07
Mean of eight determinations involving 0·1 % glucose								**4·0**	**−6·2**	**−0·02**

Sheep. The mean $-Q_{O_2}$ was slightly greater for the combined substrates than for acetate (Table 6); in seven experiments it was greater, approximately the same in two and slightly less in two (Table 5). The mean $-Q_{acid}$ for both substrates together was somewhat higher than for acetate alone (Table 6), this effect being apparent in seven of the eleven experiments (Table 5). R.Q. in the combined substrates was greater than in acetate (Table 6); this held for nine of the eleven experiments shown in Table 5. Therefore, in the sheep also, glucose stimulates acetate utilization by mammary gland slices.

DISCUSSION

The finding that lactating mammary gland slices from ruminants (cow and goat) fail to utilize glucose with R.Q. > 1, previously reported (Folley & French, 1949*c*), has been confirmed and extended to sheep in early lactation. There is therefore now no question of the indifference of ruminant mammary tissue to glucose (leaving out of account possible lactose synthesis from glucose) being due to the use of tissue taken in late lactation in which the lactation potential is low; it is clear that we are dealing with a real metabolic difference between mammary tissue from ruminants on the one hand and from non-ruminants (mouse, rat, guinea pig, rabbit) which utilizes glucose as sole substrate with high R.Q. on the other.

The difference is further emphasized by the fact that the situation is reversed when acetate is used as sole substrate. Lactating tissue from ruminants, whether taken in early or late lactation, markedly utilizes acetate with R.Q. > 1, while slices from fully lactating non-ruminants (rat, rabbit) show a low R.Q. in acetate. However, mammary tissue from

Table 5. *Effect of glucose on utilization of acetate by lactating mammary gland slices from sheep*

Sheep no.	Stage of lactation (days)	Acetate (0·02 M)			Glucose (0·3 %)			Acetate (0·02 M) + glucose (0·15 %)			(Acetate + glucose) − (acetate)		
		$-Q_{O_2}$	Q_{acid}	R.Q.	$-Q_{O_2}$	Q_{acid}	R.Q.	$-Q_{O_2}$	Q_{acid}	R.Q.	$\Delta -Q_{O_2}$	ΔQ_{acid}	Δ R.Q.
1	34	6·7	−5·0	1·19	4·1	2·3	0·88	8·6	−5·7	1·38	1·9	−0·7	0·19
2	9	7·2	−4·0	1·10	4·7	2·0	0·80	8·0	−6·3	1·40	0·8	−2·3	0·30
2	16	4·7	−3·0	1·10	3·9	1·8	0·98	8·1	−8·0	1·58	3·4	−5·0	0·48
2	23	6·5	−4·2	1·20	2·7	1·5	0·92	8·5	−4·9	1·44	2·0	−0·7	0·24
2	34	3·1	−0·4	1·01	2·0	2·9	0·69	2·7	1·0	0·90	−0·4	1·4	−0·11
3	10	6·6	−5·1	1·28	4·2	1·2	0·99	6·7	−5·2	1·49	0·1	−0·1	0·21
3	17	5·2	−3·2	1·22	2·9	2·2	0·74	7·0	−6·7	1·63	1·8	−3·5	0·41
3	24	4·9	−3·6	1·26	2·4	2·4	0·69	4·0	−2·1	1·27	−0·9	1·5	0·01
3	32	8·3	−7·5	1·44	4·1	2·3	0·97	10·8	−9·8	1·62	2·5	−2·3	0·18
3	43	9·3	−5·9	1·18	5·2	1·1	1·05	9·4	−9·4	1·65	0·1	−3·5	0·47
4	10	5·5	−2·7	1·10	3·8	2·9	0·85	6·3	−2·3	1·14	0·8	0·4	0·04
										Mean	1·1	−1·3	0·22

Table 6. *Effect of glucose on the utilization of acetate by lactating mammary gland slices*

Substrate	No. of determinations	$-Q_{O_2}$ Mean	$-Q_{O_2}$ Range	Q_{acid} Mean	Q_{acid} Range	R.Q. Mean	R.Q. Range
Rats: 14–21 days in lactation, seventeen animals used in all							
Glucose (0·1 %)	5	8·2 ± 0·3	7·6– 9·1	0·7 ± 0·3	0·2–1·8	1·32 ± 0·05	1·22–1·49
Acetate (0·02 M)	10	5·3 ± 0·3	3·9– 7·3	0·2 ± 0·3	(−0·9)–1·2	0·82 ± 0·04	0·60–0·94
Glucose (0·1 %) + acetate (0·02 M)	8	9·7 ± 0·4	8·2–10·9	−2·3 ± 0·3	(−1·1)–(−3·5)	1·32 ± 0·04	1·15–1·51
Rabbits: 2–5 weeks in lactation, twenty-six animals used in all							
Glucose (0·1 %)	8	5·4 ± 0·4	4·0– 7·5	1·2 ± 0·1	0·6–1·8	1·30 ± 0·05	1·04–1·49
Acetate (0·02 M)	9	6·8 ± 0·4	4·9– 9·0	−2·5 ± 0·4	(−1·0)–(−4·4)	0·92 ± 0·05	0·74–1·18
Glucose (0·1 %) + acetate (0·02 M)	19	10·1 ± 0·4	7·4–13·4	−5·4 ± 0·4	(−2·6)–(−8·4)	1·25 ± 0·03	0·91–1·63
Sheep: 7–47 days in lactation, four animals used in all							
Glucose (0·3 %)	15	3·9 ± 0·3	2·0– 5·2	1·9 ± 0·2	0·7–2·9	0·88 ± 0·01	0·69–1·05
Acetate (0·02 M)	15	6·1 ± 0·3	3·1– 9·3	−4·1 ± 0·5	(−0·4)–(−7·5)	1·17 ± 0·04	0·93–1·44
Glucose (0·15 %) + acetate (0·02 M)	11	7·3 ± 0·3	2·7–10·8	−5·4 ± 0·8	1·0–(−9·8)	1·41 ± 0·07	0·90–1·65

The results with all three species were essentially similar as regards the effect of glucose on acetate metabolism. In the rat, mammary tissue which is practically inert towards acetate, will utilize it in presence of glucose without any decrease in R.Q. below the value in glucose; in the rabbit glucose increases the already appreciable acetate utilization, again with maintained R.Q.; while in the sheep, glucose again increases, but relatively slightly, the already considerable acetate uptake, but in this case the R.Q., already > 1, is raised still further.

non-ruminants is not necessarily inert towards acetate, because, while this is more or less true for the rat, rabbit tissue does show an appreciable utilization of acetate, but with R.Q. < 1.

As we have previously pointed out (Folley & French, 1949*c*), it seems reasonable to interpret a high R.Q. obtained under these conditions as indicating net synthesis of fat by the tissue slice, in which case these experiments indicate that ruminant mammary gland is adapted for the synthesis of fat from acetate alone, while non-ruminant mammary tissue is virtually unable to utilize acetate alone for fat synthesis (rat) or at any rate only at a speed perhaps sufficient to give a turnover, detectable by means of tracer isotopes, but not a net synthesis manifested by R.Q. > 1 (rabbit). In this connexion the known facts regarding the importance of exogenous acetate in ruminant metabolism (Elsden & Phillipson, 1948) are surely of significance. That similar considerations probably apply to other herbivores which may produce acetate in the caecum (Elsden, Hitchcock, Marshall & Phillipson, 1946) should be noted in regard to our findings in the rabbit.

Our present results, at any rate as far as ruminants are concerned, provide the first experimental evidence (first reported in preliminary form by Folley & French, 1948*a*) in favour of the theory (Folley, 1945), that acetate is utilized by the mammary gland as an important precursor of milk fat, a theory which would explain the high *in vivo* R.Q. for the ruminant udder. Results for the cow which further support this theory have since been obtained by McClymont (1949), who has observed appreciable A.V. differences in blood acetate across the udder.

Additional support for this theory has recently come from tracer experiments on the pregnant rabbit. It is generally believed that the secretory phase in the mammary gland begins in mid-pregnancy (Turner, 1939) and this is confirmed as regards fat synthesis by the observations of Popják & Beeckmans (1949*a*) who found a high rate of incorporation of deuterium into glyceride fatty acids isolated from the mammae of 28-day pregnant rabbits receiving heavy water. The radioactivity of glyceride fatty acids from pregnant rabbits given carboxyl-labelled sodium acetate ($CH_3{}^{14}COONa$) was much higher than that of fat from foetal tissues or from the maternal liver (Popják & Beeckmans, 1949*b*) which strongly suggested the rapid utilization of acetate by the mammary gland itself for milk-fat synthesis even before parturition. It is, of course, probable that some of the fat isolated from the mammae during pregnancy consists of depot fat, but it is certain that a good proportion must have been milk fat since short-chain acids, characteristic of milk fat and absent from depot fat, have been isolated from these fat samples (see above).

Since these tracer studies indicate that active fat synthesis from acetate is already proceeding in the rabbit mammary gland before parturition, and since the initiation of copious lactation results in a striking acceleration of this process, as indicated by the increased respiration and rise of R.Q. above unity observed with rat mammary gland slices incubated with glucose (Folley & French, 1949*d*), the low R.Q. exhibited in acetate by mammary slices from *lactating* rabbits is at first sight somewhat surprising. It must be remembered, however, that *in vitro* experiments involving one substrate only may have only a limited analogy to *in vivo* conditions in which a wider range of substrates is available.

Our demonstration that non-ruminant mammary tissue will vigorously utilize acetate in presence of glucose with R.Q. > 1 does much to harmonize our results on mammary gland slices with the *in vivo* experiments of Popják & Beeckmans (1949*a*, *b*), since glucose is available to the gland in the intact animal. Of the two species studied, the effect was more striking in the rabbit in which, on the average, the addition of glucose to 0·1 % caused a decrement in Q_{acid} amounting to $-6{\cdot}2$ (Table 4), the high R.Q. (in 0·1 % glucose) being maintained. The mean increment in $-Q_{O_2}$ amounted to 4·0, which, assuming that glucose is also being oxidized, is quite insufficient to account for the increased acetate utilization in terms of oxidation, which would require $-Q_{O_2}$ to increase by 12·4. The explanation seems inescapable that most of the extra acetate has been utilized for fat synthesis. In the case of the rat the results (Table 3) were qualitatively similar, but the effect not so striking, the mean decrement in Q_{acid} in four experiments amounting to $-2{\cdot}7$. Here again the extra oxygen consumption would not suffice for oxidation of the acetate utilized if the glucose were also oxidized. The R.Q. with the mixed substrates was again hardly changed from the value obtained in 0·1 % glucose, so that it appears that even rat mammary tissue, which is virtually inert towards acetate *in vitro*, can utilize it for fatty-acid synthesis in presence of glucose.

With sheep tissue, which is almost indifferent to glucose, but vigorously utilizes acetate, the addition of glucose to 0·15 % causes a small increase in the acetate uptake above that observed with acetate alone, with a small increase in respiration. The R.Q., however, is appreciably increased above the already high value in acetate (Tables 5 and 6). Thus it may be said that in ruminant and non-ruminant alike, glucose stimulates the utilization of acetate by the mammary gland for milk-fat synthesis.

It must be noted, however, that mammary tissue normally contains small amounts of glycogen (Folley, 1949), and in unpublished determinations we have found rather more in ruminant gland than in tissue from non-ruminants. It is possible, therefore,

that the apparent difference between mammary tissue from these two types of mammal in the ability to effect fatty acid synthesis from acetate alone is a reflexion of differences in the carbohydrate stores of the tissue. It is true that our results do not exclude the possibility that ruminant mammary tissue devoid, or nearly devoid, of glycogen might be inert in presence of acetate alone, notwithstanding the fact that addition of glucose stimulates still further the incorporation of acetate into fat by ruminant mammary gland slices as indicated by an increase in R.Q. In any case, since Q_{O_2} and R.Q. in glucose alone are low, the most striking effect of glucose on the metabolism of ruminant mammary slices, which we have found so far, is the stimulation of acetate utilization. Therefore, although acetate metabolism may follow a similar course in ruminant and non-ruminant mammary tissues, it appears from these experiments that acetate and not glucose is the specific requirement for the synthetic reactions in the ruminant udder which result in a high R.Q.

If it should transpire that likewise in other ruminant tissues acetate, the characteristic product of carbohydrate breakdown in the rumen, is required for metabolic purposes subserved in other animals by blood glucose, these present experiments may have wider implications for an understanding of ruminant biochemistry.

It may be noted that the stimulating effect of carbohydrate on fat synthesis from acetate by liver slices, observed by Bloch & Kramer (1948), was only manifested by an increased turnover of fat, which can only be detected by means of tracer isotopes. In our experiments the effect is of such a magnitude as to be observable by classical manometric techniques, a fact which, besides indicating the essential similarity of the mechanism of fat synthesis in mammary gland and liver, reinforces our earlier contention (Folley & French, 1949*c*) that lactating mammary tissue is peculiarly suitable for *in vitro* studies of fat synthesis.

The mechanism of the stimulating effect of glucose on fat synthesis from acetate by mammary tissue must be further studied, though a few possibilities should be pointed out. The gland of the non-herbivore seems unable to oxidize acetate, and carbohydrate may be necessary to provide energy without which fat synthesis cannot proceed, as suggested by Bloch (1948) in connexion with fat synthesis by liver slices. Carbohydrate may also be required as a source of additional carbon for fatty acid synthesis. Endogenous acetate is, of course, available *in vivo* in the rat from the acetate pool, but Bloch (1947) calculates that only some 25% of the fat synthesized in the body of the rat comes from this source, the rest of the carbon coming presumably from carbohydrate via pyruvate (Bloch, 1948). Though our results indicate that in the ruminant mammary gland, acetate can function not only as the sole source of carbon for fatty acid synthesis but also as a source of energy enabling the synthesis to proceed, it is not unlikely that here too, glucose might help matters by providing additional carbon. Yet another possible role for glucose is worth mentioning. It may provide the glycerol necessary for the synthesis of the glycerides which form the bulk of the milk fat. In a few unpublished experiments we have found that glycerol, under certain conditions, stimulates the utilization of acetate by lactating mammary slices.

In conclusion, it may be well to emphasize that it is not suggested that the whole of the milk fat is synthesized in the mammary gland from acetate or carbohydrate, since the A.V. studies, already mentioned, show clearly that the lactating udder of the ruminant takes up considerable quantities of neutral fat from the blood, much of which must be transformed into milk fat.

SUMMARY

1. The respiration and the production or utilization of acid by lactating mammary gland slices from ruminants (sheep, goat, cow) and non-ruminants (rat, rabbit) have been studied with acetate and/or glucose as substrates.

2. Mammary tissue from ruminants, whether in early or late lactation, is by contrast with tissue from non-ruminants almost inert towards glucose as shown by low Q_{O_2} values and R.Q. < 1.

3. Mammary slices from lactating ruminants markedly utilize acetate, Q_{O_2} being greater than values without substrate or in glucose, and R.Q. > 1. On the other hand, tissue from non-ruminants shows R.Q. < 1 in acetate, though some acetate is utilized by rabbit tissue and Q_{O_2} is as great as in glucose.

4. Mammary slices from a well-lactating sheep showed a regular increase in $-Q_{O_2}$, R.Q. and acetate utilization ($Q^{O_2}_{acid}$) over the period 7–47 days post-partum, i.e. over the period of increasing milk yield. In glucose $-Q_{O_2}$ and R.Q. were the same as in acetate at the outset, but showed no subsequent increase.

5. Glucose stimulates acetate utilization by mammary slices from rat, rabbit and sheep. In presence of glucose, rat mammary tissue utilized some acetate with R.Q. > 1 and indeed similar to the value in glucose alone; in rabbit tissue acetate utilization was much increased, again with the high R.Q. maintained; while in the sheep the main effect of glucose was to increase R.Q. over the already high value for acetate alone. In the rat and rabbit the increment in Q_{O_2} was insufficient to account for the increased acetate uptake in terms of oxidation.

6. The results are regarded as evidence of the utilization of acetate for milk-fat synthesis by the mammary gland, and they are discussed from this point of view.

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A Chemical Study of Rabbit Antiovalbumin

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There are three main theories purporting to explain the formation of antibodies in an animal in response to the injection of an antigen. All accept the principle that combination of antibody with antigen depends on the configuration of parts of the antibody molecule being complementary to parts of the antigen molecule. The theory due to Breinl & Haurowitz (1930) and Mudd (1932) postulated that the specific configuration of the antibody arose because the antigen acted as template around which the protein-synthesizing system made new antibody. Burnett (1941) offered criticism of the theory and suggested that the protein-synthesizing system itself, was modified by contact with antigen. Pauling (1940) brought forward a different theory, in which the formation of antibody was regarded as due to refolding of preformed γ-globulin, in the presence of antigen, into a new configuration complementary to that of the antigen. An apparently successful attempt to demonstrate this mode of formation *in vitro* (Pauling & Campbell, 1942) has not been confirmed (Campbell, 1948); moreover, the theory fails to explain many characteristics of immunity (Burnett, 1941) such as: (1) the persistence of antibodies in plasma for considerable periods after the last known contact with antigen; (2) the difference between primary and secondary response to injection of antigen; (3) the increase in antibody titre after bleeding of the animal; (4) the increase in concentration of non-specific serum globulin during immunization; (5) the evidence that the site of antibody formation is localized; (6) the demonstration that antibody can retain its specific action after denaturation (Erickson & Neurath, 1945).

Pauling's theory postulates only a change in the physical state during transformation of γ-globulin

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into antibody, whereas other theories permit a difference in chemical composition between inert γ-globulin and antibody globulin. A comparison of the chemical structure of antibody with that of associated globulin should provide some evidence by which to judge these theories. In the past, a variety of amino-acid analyses of antibodies and various globulin fractions from normal and immune sera have been carried out (Calvery, 1935; Smith, 1946; Smith, Greene & Bartner, 1946; Velluz, 1934). Though no doubt most of these could be criticized on the grounds of analytical technique or purity of material, the general conclusion seems justified that few, if any, differences exist in overall amino-acid composition between the active and inactive material. It is, however, possible that proteins of very similar amino-acid content could differ greatly in general structure, and advantage has therefore been taken of other techniques to investigate this problem further. In particular, end-group assay has been used to estimate the number of open polypeptide chains and the identity of the amino-acids in the terminal sequence has been established. Although only the nature of the terminal pentapeptides was established, the possibilities of variation in this fragment are so great that some inference as to comparative structure of the rest of the inert γ-globulin and antibody globulin may be drawn.

Electrophoretically pure γ-globulin was prepared from sera of rabbits strongly immunized with ovalbumin, and the chemical properties of the fraction (about one-third) combining with antigen were compared with those of the immunologically inert fraction. This seemed preferable to comparing the properties of pure antibody prepared by dissociation of specific precipitate (Heidelberger & Kendall, 1936; Haurowitz, Tekman, Bilen & Schwerin, 1947) with the γ-globulin from normal rabbits.

METHODS

Immunization of rabbits. The animals were given, twice weekly, intravenous injections of 0·5 ml. of a 5% (w/v) solution of crystalline ovalbumin in isotonic saline, for 6 weeks. After 10 days' rest the animals were bled, and the serum tested for activity. The most responsive animals were kept and given a second course of injections, the rest being discarded. In this way was acquired a stock of some twelve responsive animals which have been injected and bled regularly for more than 12 months.

Preparation of γ-globulin. It was found that the simple Na_2SO_4 fractionation of serum described by Kekwick (1940) for the preparation of human γ-globulin was equally successful in the preparation of γ-globulin from the sera of normal and immune rabbits. As would be expected, the yield was higher using immune sera, but in both cases electrophoretic examination showed the γ-globulin preparations to contain only traces of contaminants. Some loss of antibody occurred during the preparation, but attempts to avoid this by modifying the procedure led to electrophoretic impurity and were not pursued.

Flocculation tests. Tests were carried out under standard conditions in a 45° bath with indirect lighting, the tubes being only three-quarters submerged to stimulate convection.

Reduction of S–S *bonds.* Reduction was effected by dissolving Na thioglycollate in approximately 5% γ-globulin solution to give a neutral solution, 1·1 M with respect to thioglycollate.

End-group assay. Estimation of unreactive amino and iminazole groups, and determination of the terminal peptide sequence was carried out using a technique previously described (Sanger, 1945; Porter & Sanger, 1948; Porter, 1948; Sanger, 1949; Porter, 1950*a*). A collective description of these methods has been given by Porter (1950*c*).

Ovalbumin. This was a five-times recrystallized specimen given by Prof. R. K. Cannan.

RESULTS

Ovalbumin

(The abbreviations DNP and FDNB are used in this paper for dinitrophenyl and fluorodinitrobenzene.) As a preliminary to work with the specific precipitate, the free amino groups of ovalbumin were estimated. By no method of hydrolysis could a significant amount of N^1-dinitrophenyl amino-acid be obtained from ovalbumin after reaction of the protein with 1:2:4-fluorodinitrobenzene. It is therefore concluded that ovalbumin is a cyclic molecule or alternatively, that the carbohydrate, known to be present, is condensed on the N^1-amino groups. As the carbohydrate is firmly bound (Neuberger, 1938) and the N^5-amino groups of lysine react quantitatively with FDNB, the second alternative is possible.

All the iminazole groups of the histidine residues reacted with FDNB whether the protein was native or denatured (Porter, 1950*a*).

γ-Globulin

End-group assay was carried out on γ-globulin prepared from normal and immune sera when native and denatured. The specific precipitate and the inert γ-globulin, left in solution after precipitation of the antiovalbumin were also examined. In working with specific precipitate the sodium bicarbonate concentration during reaction with FDNB was lowered to 4%, as this was sufficient to maintain the pH at 8·5 and lessened the risk of dissociating the complex. Unfortunately no decisive evidence could be obtained as to whether splitting of the complex occurred during the reaction; however, the complex was insoluble in the sodium bicarbonate solution and remained so on addition of the ethanolic solution of FDNB. It is therefore unlikely that dissociation of the precipitate occurred. In calculating the result in terms of γ-globulin, allowance was made for the weight of ovalbumin present. This was estimated as described (Porter, 1950*b*). The results of these estimations are summarized in Table 1.

Table 1. *End-group assay on rabbit γ-globulin by reaction with FDNB*

Protein	Terminal amino-acid	Mol. of terminal amino-acid/protein unit of mol. wt. 160,000		No. of reactive lysine N^5-amino groups/protein unit of mol. wt. 160,000	
γ-Globulin from normal rabbit	Alanine	1·1	1·2	66	70
γ-Globulin after acid denaturation	Alanine	1·2	—	77	—
γ-Globulin from immune rabbit	Alanine	1·0	0·9	68	71
Immune γ-globulin after absorption of antibody	Alanine	1·0	1·1	73	69
Immune γ-globulin in 6M-guanidine	Alanine	1·0	—	70	—
Specific precipitate antibody	Alanine	1·0	1·0	70	75
Specific precipitate in 6M-guanidine	Alanine	1·1	—	70	—

It would seem that all the γ-globulin preparations and fractions examined, whether immunologically active or not, are identical in having one terminal alanyl residue per molecule. This presumably means that γ-globulin, if homogeneous, is a single polypeptide chain of some 1500 amino-acid residues. The possibility of cyclic structures being present cannot be ignored, but seems improbable in view of the results obtained with papain-HCN hydrolysis (Porter, 1950*b*). This treatment split the molecule, to give an active fragment having one alanyl terminal residue per unit of mol. wt. 40,000 and other fragments, none of which had this terminal residue. The active fragment was presumably the terminal quarter of the polypeptide chain and as papain-HCN produces quarter molecules from γ-globulin (Petermann, 1946) it seems unlikely that a cyclic configuration is included in the structure.

It is evident from the results given in Table 1 that the combination of antibody and antigen in the specific percipitate did not result in the blocking of the N^1-amino group or of any significant quantity of N^5-amino groups.

The normal and immune γ-globulins and specific precipitate were tested for unreactive iminazole groups with the results shown in Table 2. Comparison of these results with those obtained with other proteins (Porter, 1950*a*) shows that in normal and immune γ-globulin the iminazole rings are in a reactive state. The specific precipitate failed to react with FDNB to the same extent when parallel experiments were run, and the difference is probably significant. Such a result indicates that one iminazole group is blocked per molecule of γ-globulin. Alternatively, since the molecular ratio of antibody to ovalbumin and the specific precipitate is 3:1, three iminazole groups in the ovalbumin might be blocked.

Table 2. *Reactivity of the iminazole group of γ-globulin with FDNB*

γ-globulin	Histidine content (g. histidine/100 g. protein)	Unreactive histidine content (g. histidine/100 g. protein)	Unreactive histidine as percentage total histidine
Normal	2·56	0·05	2
Immune	2·56	0·07	2·5
Specific precipitate	2·56	0·17	7

Terminal peptide sequence

The terminal peptide sequence was determined by the method of Sanger (1949). Both inert γ-globulin, left after the absorption of antiovalbumin, and specific precipitate were principally used, though other fractions were also examined. As ovalbumin has no N^1-amino groups, the specific precipitate could be regarded as pure antibody if allowance was made for the weight of antigen. A weighed amount (about 0·8 g.) of air-dried DNP-γ-globulin fraction was dissolved in A.R. 12N-hydrochloric acid and incubated at 37° for 7 days.

Fractionation of partial hydrolysate. The black solution was diluted with several volumes of water and extracted four times with ethyl acetate, the extracts being washed with small volumes of water. Colour may continue to be extracted by further amounts of ethyl acetate, but it contains no N^1-DNP peptides. The ethyl acetate solution was extracted three times with 1% $NaHCO_3$, and each $NaHCO_3$ extract washed with fresh ethyl acetate. The $NaHCO_3$ solutions were combined and acidified with HCl, extracted with ethyl acetate, and the extracts washed with N-HCl, combined and concentrated. With DNP-insulin this procedure separated N^5-DNP-lysyl peptides from N^1-DNP peptides, but with DNP-γ-globulin, where the ratio of lysyl to alanyl peptides is about 100:1, complete separation was not effected and some DNP-lysyl peptides reached the final ethyl acetate extract.

Chromatographic separation of DNP peptides. The combined ethyl acetate extracts containing N^1-DNP peptides were taken to dryness, redissolved in wet 15% butanol chloroform (*B*15) and put on a *B*15 wet silica column. The fast band was taken to dryness and re-run on a *B*5 column, the fast band was again taken to dryness and re-run on a chloroform column. The fast moving band from the *B*15 column, when run on a *B*5 frequently tailed badly, but again moved fast or with $R=1$ (R has been defined by Martin & Synge, 1941). The slow moving bands from the *B*15 columns, which were lysyl peptides, were discarded. On the *B*5 column the slow moving band was shown to contain N^5-DNP-lysine and was also discarded. On the chloroform column there was a trace of fast moving coloured

material, a band moving at a rate of about $R0{\cdot}5$ (A_1) another $R0{\cdot}2$ (A_2), and slower moving colour which on development with $B1$ resolved into three distinct bands, $R0{\cdot}3$ (A_3), $R0{\cdot}15$ (A_4) and $R0{\cdot}1$ (A_5). In addition, there was a little colour which moved very slowly on $B1$ columns, and was stationary on chloroform columns. This colour was present on complete hydrolysis, when DNP specific precipitate or when the DNP derivative of inert γ-globulin was used. It could not be identified as a DNP amino-acid and on hydrolysis did not give rise to an amino-acid. This suggests that it is not a peptide and is probably an artefact.

Identification of terminal peptide sequence. The different bands were collected and estimated colorimetrically (Porter, 1950*c*). The yield of each peptide was estimated by reference to a standard curve of DNP-alanine and the results are expressed as mol. of peptide per mol. of protein (Table 3).

Table 3. *Yields of terminal DNP peptides from partial hydrolysis of DNP-γ-globulin and DNP specific precipitate*

	Mol./unit of mol. wt. 160,000	
Peptide	Inert globulin	Antibody globulin of specific precipitate
DNP alanine	0·37	0·40
DNP A_1	0·29	0·25
DNP A_2	0·22	0·23
DNP A_3	0·15	0·17
DNP A_4	0·09	0·11
Total	1·12	1·16

The material from each band was extracted into organic solvent, taken to dryness, redissolved in a very small volume of 6N-HCl and the solution sealed in a capillary tube for 24 hr. hydrolysis at 105°. Identification of the amino-acids in the hydrolysate was by partition chromatography on paper, using phenol-water in a coal gas NH_3 atmosphere as the principal solvent system. Butanol-acetic acid (Partridge, 1948) and butanol-benzyl alcohol (Consden, Gordon & Martin, 1944) were also found to be useful solvent systems. The DNP amino-acid present was identified by extracting the hydrolysate with a small volume of ether and running the ether-soluble material on silica-gel columns in the usual manner.

The results obtained are given in Table 4. In addition to the amino-acids listed in Table 4 much smaller quantities of alanine were also frequently found and are believed to have arisen from the partial decomposition of DNP-alanine during hydrolysis. The presence of two distinct peptides A_4 and A_5 with the same constituent amino-acids must arise, in view of the composition of the smaller peptides, from the presence of an amide group on the aspartyl residue in one of them. It is highly probable therefore that the terminal tetrapeptide is alanyl-leucyl-valyl-asparaginyl. In order to confirm this, it was desirable to isolate sufficient quantities of the tetrapeptides, to enable the result of their partial hydrolysis under similar conditions to be studied. Attempts to achieve this by using shorter times of hydrolysis (4–6 days) were not successful; the only result was the production of smaller quantities of all these peptides, together with higher peptides. However, two pentapeptides moving very slowly on a $B3$ column, or at nearly $R=1{\cdot}0$ on a $B5$ column, were isolated and examined. Both contained, in addition to the amino-acids previously found, glutamic acid, and it is probable therefore that this is the fifth amino-acid in the sequence.

Table 4. *Amino-acid composition of DNP peptides from DNP-γ-globulin*

Peptide	
A_1	DNP-alanine, leucine
A_2	DNP-alanine, leucine, valine
A_3	DNP-alanine, leucine, valine, aspartic acid
A_4	DNP-alanine, leucine, valine, aspartic acid

Throughout this work no distinction could be found between the products of partial hydrolysis of the inert DNP-γ-globulin and the DNP specific precipitate.

Reduction of antibody globulin with sodium thioglycollate

From the previous work it seemed probable that there was considerable similarity, in the amino-acid sequence of the polypeptide chain in all fractions of γ-globulin. The effect of reduction of S–S bridges with thioglycollate was therefore examined, as in the absence of other stable intrachain bonds, this might be expected to have a pronounced effect on the molecular configuration.

Blumenthal (1936) showed that reduction of ovalbumin with M-thioglycollate had no influence on its antigenic behaviour. This was confirmed and it was also noted that oxidation of the SH groups present in this protein, by titration with iodobenzoate (Bailey & Perry, 1947) had no influence on its combination with antibody, though complete removal of SH groups was confirmed by the absence of a nitroprusside reaction in guanidine solution.

The effect of sodium thioglycollate on the immune γ-globulin was examined qualitatively as follows.

Immune γ-globulin solution (1 ml. of approx. 10%) was mixed with 1 ml. of a neutral 2·2M-solution of Na thioglycollate at room temperature. After different time intervals 0·1 ml. samples of the mixture were removed and diluted to 5 ml. with saline, thus reducing the thioglycollate concentration to 0·022M. Previous work had shown that this concentration had little or no effect on the flocculation of antibody and antigen, though it should be sufficient to prevent reoxidation during the period of the test. The titre and time of flocculation of the reduced immune globulin was now found by the α procedure (Boyd, 1946) using serial (50%) dilution of ovalbumin solution.

After a few minutes contact with 1·1 M-thioglycollate, the time of flocculation was increased fivefold, though the optimal proportion was unaffected. The flocculation time increased with increase in time of contact with 1·1 M-thioglycollate at a decreasing rate to reach a maximum at about 10–15 times the original value (2 hr. compared with an original flocculation time of 10 min.). This was reached after several hours' contact with reducing agent. Precipitation with antigen still occurred even after the immune globulin had been in contact with the thioglycollate for 27 hr. at room temperature. SH estimations were carried out using the ferrocyanide method (Anson, 1942), but were unsatisfactory, as, after 20 hr. reduction, they gave values exceeding the theoretical maximum for the known cystine content of globulin. If globulin, reduced for 2 hr., was dialysed free of thioglycollate (against running tap water for 24 hr.) reoxidation occurred and the increased time of flocculation was completely reversed. This reversal on dialysis was obvious though not complete after 27 hr. reduction.

DISCUSSION

The time of flocculation of different sera may vary considerably, even when the antibody concentration is the same. The factors which cause this variation are unknown. The effect of reduction with thioglycollate suggests that the time of flocculation may be influenced by the structure of the antibody. As no SH groups were detectable in the native protein, nor were demonstrable after denaturation, it seems likely that the effect of the thioglycollate consists of a reduction of S–S bonds to SH groups. It seems that the combining power of the antibody had only been slightly affected by the breaking of the S–S bridges and that reoxidation can almost completely reverse the effect. This could be explained if other stable intrachain bonds existed which held the molecule in the original configuration; alternatively the combining sites of the antibody may be so small and relatively distant from the cystine residues that they are little influenced by the rupture of the S–S bonds. It is particularly interesting to note that combining power can be kept relatively constant while the rate of flocculation is greatly altered. Apparently the connexion between combination and precipitation is not so direct as has been suggested in the lattice theory (Marrack, 1938) of the precipitin reaction.

The results which have been obtained from end-group assay suggest that antiovalbumin and the inert γ-globulin with which it is associated are both single long chain polypeptides with the terminal sequence, alanyl-leucyl-valyl-aspartyl-glutamyl. There is some evidence that an amide group may be on the β-carboxyl of the aspartic acid. No distinction could be found between the two γ-globulin fractions, nor was any evidence of heterogeneity of either fraction found. This is surprising in view of the known inhomogeneity of γ-globulin fractions from various species: thus electrophoretic (Deutsch, Alberty & Gostling, 1946), ultracentrifuge (Pedersen, 1945) and salting-out studies (Derrien, 1947) have all demonstrated the presence of more than one component. It is however, worth noting that inhomogeneity could exist even if all the materials consisted of the same amino-acids arranged in the same sequence in the polypeptide chain. It has been shown that a number of polar groups in a protein may or may not be capable of ionization, or be available to different reagents, including solvents (cf. Anson, 1945). It is clear that the failure of phenolic groups to ionize, as occurs in ovalbumin (Crammer & Neuberger, 1943), or the unavailability of amino groups, as occurs in some serum globulin (Porter, 1948), will have a marked effect on the electrophoretic mobility and solubility of the protein. The molecular weights of several proteins have been shown to depend, over a wide range, on the concentration of protein and salt in a solution. Presumably the measured molecular weight is the resultant of association and dissociation of the smallest unit (cf. Adair, Bailey & Tsao, 1949). The recent work of Northrop & Goebel (1949) on crystalline horse pneumococcus type I antibody, where solubility studies showed the crystalline material to be quite heterogeneous and also very unstable, suggests that the breaking of labile bonds such as are responsible for the unreactivity of polar groups and the association of molecules in solution, may be responsible for the apparent heterogeneity. The several components of such a mixture might even in some cases be the result of the preparative procedure. It is possible, therefore, that various changes of labile bonding of the molecule may account for the many components of γ-globulin.

In the particular case of antibody and other γ-globulin the limitations of the methods used in this work should be emphasized. Thus, with the material available, it is possible that a component representing 10 % of the total which had a different amino-acid in the fourth position would have been missed, particularly if the adjacent peptide linkage was unusually labile to the type of hydrolysis used. This might be overcome by the use of more DNP-globulin per estimation and by varying the methods of partial hydrolysis, but owing to the necessity of immunizing large numbers of rabbits, sufficient material was not available.

From the finding that few if any amino groups and only one or two iminazole groups were bound in the floccule, and that breaking the S–S bonds had little effect on combining power, it seems probable that the combining site of the antibody is small. How far the identity of the terminal amino-acid sequence in the

antibody and inert γ-globulin may be used to infer identity of sequence throughout the remainder of the molecule, is debatable. As 19^5 pentapeptides could theoretically occupy the terminal position it is clear that the similarity between the biologically active and inert fractions cannot be coincidental. It therefore seems possible that this similarity will extend to a considerable part of the whole molecules, but the combining sites which appear to be small may well have quite different composition from the equivalent section of the inert material.

The results described are in agreement with Pauling's theory of antibody formation in that no chemical distinction between the fractions could be found. According to this theory the rupture of the S–S bonds would have been expected to alter the configuration and therefore to destroy the antibody activity, but it is possible that the configuration of part of the molecule was unaffected by the reaction.

Final conclusions as to the validity of this and other theories of antibody formation must await more detailed work, but it is clear that the chemical evidence described here, contrary to the physiological evidence discussed in the introduction, is in accordance with Pauling's theory.

SUMMARY

1. End-group assay has shown that the immunologically active and inactive fractions of γ-globulin from rabbit antiovalbumin sera are alike in having one terminal alanyl residue per molecule.

2. In none of the fractions was a significant number of unreactive amino or iminazole groups detected, and they occurred only in small amounts in the specific precipitate.

3. All globulin fractions were alike in having the terminal tetrapeptide alanyl-leucyl-valyl-aspartyl with glutamic acid probably occupying the fifth position.

4. Rupture of the disulphide linkages by reduction with thioglycollate had little effect on the combining power of the antibody, but greatly increased the time of flocculation.

5. The significance of the results is discussed in terms of γ-globulin structure and theories of antibody formation.

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The Formation of a Specific Inhibitor by Hydrolysis of Rabbit Antiovalbumin

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In a comparative study of rabbit antiovalbumin and the inert γ-globulin with which it is associated (Porter, 1950) no definite difference in structure was found to which the specific biological activity of the antibody could be attributed. The active and inactive material apparently exist as single long polypeptide chains of about 1500 amino-acid residues, the terminal tetrapeptide being, in each case, alanyl-leucyl-valyl-aspartyl-. There remain a great many possibilities of variation in structure, but the results already obtained suggest that considerable similarity may be found between normal γ-globulin and antibody and that antibody may differ from normal γ-globulin only in that segment of the chain which is responsible for combination with antigen. The lattice theory of antibody, antigen combination (Marrack, 1938) postulated that at least two combining areas should be present on each molecule to enable flocculation to occur. If the antibody molecule could be split to give fragments containing one such combining area these fragments should be capable of combining with the antigen, but not of precipitating with it. They would therefore be expected to inhibit the flocculation of antibody and antigen by competing for the combining area of the antigen. As the combining centre of the antibody may be small enough to permit detailed chemical examination it seemed worth while to try to produce an inhibitor by hydrolysis of antibody.

No such attempt to prepare an inhibitor from an antibody appears to have been reported, but Landsteiner (1942) studied the complementary problem of attempting to produce an inhibitor by hydrolysis of an antigen. He was successful, but it is difficult to interpret his results. The antigen, which was degummed silk, was brought into solution by concentrated hydrochloric acid and after several precipitations was finally adsorbed on charcoal before injection. Degradation may have occurred during preparation and the nature of the material injected is uncertain. However, specific inhibition of the antisera could be effected by the addition of dialysable products from partially hydrolysed silk, and Landsteiner (1942) estimated the average molecular weights of the inhibitors as being 600–1000. This work suggested that the combining area of an antibody, also, might be a small fragment of similar size.

After examining a wide variety of hydrolytic conditions it has been found that rabbit anti-ovalbumin will give, on treatment with papain-HCN, a specific inhibitor of antigen-antibody flocculation which had approximately one-quarter the size of the original molecule. Subsequent treatment with carboxypeptidase reduced the size further without destroying the inhibitory activity.

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MATERIALS

No attempt was made to prepare pure antibody, but rabbit γ-globulin containing 25–35% specifically precipitating protein was used as starting material. This was prepared as described previously (Porter, 1950).

Enzyme preparations

Aminopeptidase. As a mixture of aminopeptidases with a broad specificity was required, a simple erepsin was prepared as suggested by Dr Emil L. Smith (private communication). Fresh hog gastric mucosa was ground with sand and water several times and the aqueous extracts were centrifuged. $(NH_4)_2SO_4$ was added to the opalescent solution to bring it to 40% saturation and the precipitate was discarded. The precipitate formed at 80% saturation was collected, dissolved in water and dialysed free of salt. Any precipitate formed was rejected and after adjusting to pH 8 the solution was stored at 4° under a film of toluene.

Carboxypeptidase. The enzyme was prepared from beef pancreas, according to Anson (1937*a*).

Chymotrypsin. The enzyme was a crystalline sample given by Mr D. M. Phillips.

Papain. A commercial preparation (Hopkins and Williams Ltd., London) was used without purification. It was activated by dissolving, to give a 10% solution, in 10% KCN and warming to 45° for 1 hr. The solution was centrifuged and stored for short periods at 4°.

Pepsin. A crystalline preparation was made from a commercial product according to the method of Northrop (1939).

Protease from Bacillus sporogenes. The protease was a crude preparation made according to the method of Maschmann (1938).

Trypsin. The enzyme was a crystalline sample from Armours Laboratories, Chicago.

METHODS

In attempting to find the most advantageous conditions many types of hydrolysis were investigated.

Acid hydrolysis

12N-HCl at 37° from 1 to 72 hr. Boiling N-HCl from 1 to 4 hr. Boiling 0·1N-HCl from 1 to 6 hr. 0·05M-dodecylsulphonic acid at 37° from 4 to 72 hr. 0·025M-dodecylsulphonic acid at 20° from 1 to 4 hr.

Enzymic hydrolysis

Aminopeptidase. Hydrolysis was carried out in phosphate buffer, pH 8 at 37° and in the presence of 0·001M-Mn^{+++}. Assay of the enzyme was made using L-leucylglycine and L-leucyl-glycyl-glycine as substrates (Grassmann & Dyckerhoff, 1928). As a mixture of enzymes was deliberately used this method of assay with two substrates was probably of doubtful value. When attempts at prolonged hydrolysis were made fresh enzyme was added daily as the preparation was unstable at 37° in the presence of manganese.

Carboxypeptidase. Hydrolysis was effected at 37° in phosphate buffer, pH 8·5. Enzymic assay was made using a peptic hydrolysate of edestin as substrate, as described by Anson (1937*b*).

Chymotrypsin. Hydrolysis was at 37° in phosphate buffer pH 8.

Papain. Papain-HCN solution was added to the γ-globulin solution in phosphate buffer, pH 8 at 45° for 72 hr.

Pepsin. N-HCl was added to γ-globulin solution to reduce to pH 3 or 1·5. Crystalline pepsin was dissolved in 0·1N-HCl and added to the solution. Enzymic hydrolysis was carried out at 37°, for periods varying from 1 to 72 hr.

Protease from Bacillus sporogenes. γ-Globulin was incubated with the crude enzyme preparation for 16 hr. in phosphate buffer, pH 7·4, at 37°. The activity of the enzyme was tested under similar conditions using casein as substrate, and titrating in boiling 90% ethanol till alkaline to thymolphthalein as described by Willstätter, Waldschmidt-Leitz, Dünaiturria & Künster (1926).

Trypsin. Tryptic digestion was carried out in phosphate buffer, pH 7·4, at 37°.

Testing of the hydrolytic products for inhibitory activity

The hydrolytic products were in all cases neutralized, and if necessary dialysed, before testing for activity. Their ability to flocculate with ovalbumin was tested against serial dilutions of the antigen at 45°. If this was negative, their inhibitory power was tested by adding increasing amounts of the hydrolytic products to a known amount of egg albumin solution. Sufficient antiovalbumin γ-globulin solution, which as previously determined caused the most rapid flocculation, was added to all the tubes, and the effect of the hydrolysate on the rate of flocculation found. A blank tube without inhibitor was always included in the series. A delay in the time of flocculation caused by addition of hydrolytic solution was not considered significant, unless increasing amounts of the inhibitor caused increasing delay and finally completely prevented flocculation over, say, 24 hr. When such significant inhibition was found, the possible effect of non-specific factors was tested by adding similar increasing amounts of inhibitor to a different antibody-antigen flocculating system. Rabbit anti-horse sera were normally used for this purpose.

Quantitative assay of the antibody content of the γ-globulin preparations was carried out as follows: the optimal proportions were found by the α-procedure (Boyd, 1946) which involves serial dilutions of antigen. With these sera the optimum fell in the equivalence zone. Flocculation was allowed to continue for 1 hr. at 45° and overnight at 4°. The precipitate was centrifuged and washed three times with ice-cold saline, before estimation of total N in a micro-Kjeldahl apparatus.

End-group assay of the hydrolytic products was carried out as described by Sanger (1945) and Porter & Sanger (1948).

RESULTS

The results of this survey of the effects of different conditions of hydrolysis on immune γ-globulin, may be summarized by classifying into three groups, according to the biological activity of the end products.

Hydrolytic conditions after which flocculation occurred. After several types of treatment antibody was still capable of flocculation in presence of ovalbumin. These were tryptic digestion for varying periods of time; peptic digestion at pH 3 for 16 hr.; digestion with protease from *B. sporogenes* for 16 hr.; digestion with 12N-HCl for 1 hr.

Hydrolytic conditions after which no activity remained. When antibody was subjected to the following series of treatments it was incapable of flocculation with antigen or of prevention of flocculation of antibody with antigen: chymotrypsin digestion for 12 hr.; 12N-HCl for more than 2 hr.; boiling N-HCl for 2 hr. or longer; boiling 0·1N-HCl for 2 hr. or longer; 0·05M-dodecylsulphonic acid for 2 hr. or longer; 0·025M-dodecylsulphonic acid for 2 hr. or longer.

Hydrolytic conditions after which inhibitory power appeared. Only with papain-HCN and peptic hydrolysis was there any suggestion of the successful production of an inhibitor. Production of inhibition by peptic hydrolysis for 16 hr. at pH 1·5 was not always reproducible, and a very approximate estimation of particle size, by end-group assay after partial purification of inhibitor by salt fractionation, suggested that the active principle still had a molecular weight of about 100,000. The method was therefore abandoned in favour of papain-HCN hydrolysis which gave more satisfactory results.

Inhibitor produced by papain-hydrogen cyanide hydrolysis

(The abbreviation DNP is used in this paper for 2:4-dinitrophenyl.) The conditions of papain hydrolysis finally adopted were as follows.

An approximately 5% solution of immune γ-globulin in 0·1M-phosphate buffer, pH 8, was incubated at 45°, and to it was added one-tenth its volume of papain-hydrogen

cyanide solution prepared as described. Hydrolysis was allowed to go to completion, 72 hr. being required. The hydrogen cyanide appeared to be a satisfactory antiseptic. To this hydrolytic mixture, after dialysis, anhydrous sodium sulphate was added to 15% (w/v) concentration. The precipitate, which might be expected to contain any unchanged γ-globulin, had little inhibitory power and was discarded. The further precipitate formed by raising the sodium sulphate concentration to 18% (w/v), inhibited strongly and was collected. The precipitate, formed by raising the salt concentration still higher, had little activity. The active precipitate was dialysed and fractionated further with ethanol at $-5°$. The material insoluble in 50% (v/v) ethanol was inactive, while that thrown out on raising the ethanol concentration to 75% contained most of the activity.

End-group assay was carried out on the inhibitor produced by papain-hydrogen cyanide hydrolysis and on the inactive fractions. The active fraction had alanine as terminal amino-acid residue, and as judged by this criterion was about 90% pure as only traces of other coloured derivatives were present. No significant quantities of DNP-alanine could be found in the hydrolytic products of the DNP derivatives of the inactive fractions. In the active fraction about one terminal alanyl residue was present per unit of molecular weight 40,000, whereas in the original γ-globulin only one alanyl residue was found per unit of molecular weight 160,000 (Porter, 1950). Although end-group assay is inadequate as a test for homogeneity or of molecular size, it seems probable that the inhibitor represents the terminal quarter of the original polypeptide chain. The belief that the terminal alanyl residues in the inhibitor and in the original molecule are identical, is supported by the absence of alanine as an end group in the inactive fractions. It is probable that the purified inhibitor was mixed with similar but inactive terminal quarter molecules, which had arisen from the inert γ-globulin in the starting material. Petermann's finding that papain splits human γ-globulin into quarter molecules, as measured by ultracentrifuge studies (Petermann, 1946), agrees with the approximate molecular weight of 40,000 found for inhibitor.

Further hydrolysis of inhibitor. Attempts were now made to reduce the size of the inhibitor by treatment with other enzymes and acid without destroying its activity. No success was achieved with pepsin, trypsin and 12N-HCl, but carboxypeptidase hydrolysed the molecule slowly and after 72 hr. activity was still retained. Examination of the dialysate after hydrolysis, showed that all types of amino-acid were being liberated by this enzyme. Further treatment with carboxypeptidase, however, rapidly destroyed the activity, and it was believed that the combining centre of the inhibitor had then been reached. Repeated attempts were therefore made to remove amino-acids from the other end of the chain with aminopeptidase and reduce the molecule to a still smaller size without destroying the activity. The dialysate of the hydrolytic mixture continued, after days, to give a positive ninhydrin test suggesting that amino-acids were being liberated. On one occasion a dialysable inhibitor was apparently obtained, but this result could not be reproduced. The activity of the product was unaltered by treatment with aminopeptidases, but substantial reduction in size could not be effected.

Hydrolysis of specific precipitate. In an attempt to simplify purification by using purer starting material, the specific precipitate, containing about 90% antibody and 10% egg albumin, was hydrolysed with papain-HCN as before. The hydrolysate had no inhibitor activity and on testing the hydrolysate of egg albumin alone, it was found that this had inhibitory power. It is believed that papain-HCN hydrolysis does not destroy the combining centre of antibody or antigen and leaves the two potential inhibitors combined, and hence inactive when specific precipitate is used as starting material.

The properties of the inhibitor

For study of its properties, the inhibitor was prepared as described, but purified only by salt fractionation, the ethanol fractionation being omitted. Experiments were designed to study the interaction of antibody, inhibitor and antigen, to find whether the reactions are reversible and truly competitive, as would be expected, if the inhibitor was in fact a fragment of the original antibody molecule, in which the combining centre had remained intact.

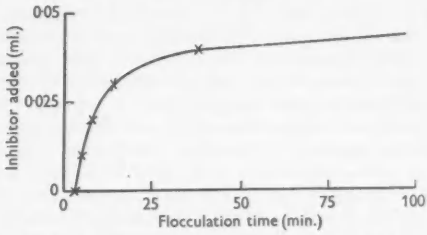

Fig. 1. Effect of increasing inhibitor concentration on flocculation rate. On addition of 0·05 ml. of inhibitor no flocculation was observed in 24 hr.

Effect of increasing inhibitor concentration. For a given solution of immune γ-globulin (1 ml. of 0·2% protein) the content of egg albumin to give the most rapid flocculation was found by serial dilution in the usual manner. Increasing quantities of inhibitor added to the antigen-antibody mixture increased the time of flocculation till none occurred even after standing in the ice chest for 72 hr. The shape of the graph (Fig. 1) and the failure to show even opalescence after a long time suggested that inhibition was in fact complete if sufficient material was added.

Table 1. *Effect of concentration of inhibitor on the weight of specific precipitate*

Exp. no.	Vol. of inhibitor solution added (ml.)	Wt. of egg albumin (mg. N)	Time of flocculation (min.)	Wt. of ppt. (mg. N)	Wt. of antibody (mg. N)
1	0	0·07	0·5	0·54	0·47
	0·4	0·07	20	0·49	0·42
2	0	0·017	1	0·148	0·131
	0·02	0·017	2	0·132	0·115
	0·08	0·017	4	0·126	0·109

A quantitative examination was made of the precipitate formed in the presence of inhibitor to determine whether the inhibitor was carried down when a precipitate was eventually formed, as happens with naturally occurring incomplete antibodies. Table 1 gives the results from two experiments; the precipitation was carried out at the optimal proportions which were in the equivalence zone.

As the inhibitor was not pure the relative content of inhibitor and antibody in the precipitate could not be found. From the data given in Table I it is apparent that less precipitate is formed in the presence of inhibitor than in its absence; it follows that inhibitor had not been carried down with the precipitate unless a considerable quantity of antibody had been left in solution. Owing to the presence of some inhibitor in the supernatant it was impossible to test satisfactorily for the presence of excess antibody or antigen.

Effect of varying antibody and inhibitor concentration relative to a fixed antigen concentration. An experiment was designed to test the competitive aspects of the inhibition. In enzymic experiments it can readily be established whether an inhibitor competes with the substrate or poisons essential groups of the enzyme. Owing to the complexity of the precipitin reaction, a direct quantitative test cannot be applied, but competition can be established qualitatively by studying the effects of varying proportions of the reactants competing for the antigen and the reversibility of these effects. A constant amount of antigen solution was run into a series of tubes and the times of flocculation measured after addition of varying amounts of antibody (in constant volume). This was repeated in the presence of two concentrations of inhibitor; in these experiments the antibody and inhibitor were added almost simultaneously. The results were plotted as indicated in Fig. 2.

Without inhibitor the system showed a flat optimum, as would be expected in this β-procedure with rabbit sera. With inhibitor the optimum occurred at a higher antibody concentration: thus in the presence of 0·1 ml. of inhibitor solution the antibody concentration necessary for precipitation in the minimum time was twice that required in its absence. The time of flocculation at the optimum was more than doubled. The effect of the inhibitor was not confined to binding a proportion of the antigen, otherwise the optimal proportion would have shifted to the right. The exact interpretation of such a complex system is not easy, particularly as the significance of the optimal proportions relationship in absence of inhibitor is not understood. It would be impossible to interpret quantitatively the more complex system of antigen, antibody and inhibitor. It would appear that a competitive mechanism is operative and that this would explain the shift in the zone of optimum proportions.

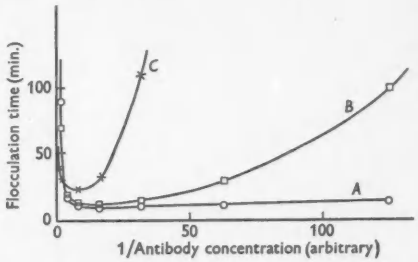

Fig. 2. Flocculation rates with varying antibody and inhibitor concentration. Constant amount of antigen throughout; ○, no inhibitor present; □, 0·02 ml. of inhibitor added; ×, 0·10 ml. of inhibitor added.

Reversibility of inhibition and precipitation. If we are dealing with competitive inhibition, the inhibition should be reversed by addition of excess antibody and conversely a specific precipitate should be capable of solution in excess inhibitor. It is apparent from Fig. 2 that, with relatively low antibody concentrations, an increase of antibody content lowers the flocculation time of a mixture containing fixed amounts of inhibitor and antigen. If the inhibitor is initially combined with antigen in the mixture, this effect would be produced by displacement of inhibitors by antibody. The forms of curves *B* and *C* are consistent with the assumption that there is a reversible equilibrium between inhibitor and antigen, such that inhibitor and antibody can compete for a limited quantity of antigen. A direct demonstration of this competition was achieved in the following manner: to a solution of antigen, inhibitor and antibody, which had failed to show any opalescence after 72 hr. in the ice chest, more antibody was added.

A precipitate formed readily; thus, a change in relative concentration had been sufficient to replace some of the antigen-inhibitor complex by antigen-antibody complex.

The reverse procedure, dissolution of the specific precipitate in excess inhibitor did not take place so readily. The solubility of the precipitate was slight and attainment of equilibrium between soluble inhibitor and precipitate was very slow; but by carrying out a quantitative experiment clear evidence of dissolution of the precipitate was obtained.

An equal volume of immune γ-globulin solution was added to each of six tubes, and to each equal amounts of egg albumin solution were added in optimal proportion. The floccules were allowed to stand at 45° for 1 hr. Two tubes were then stood at 4° overnight, the precipitate washed with cold saline three times and the N estimated in the normal manner. Two other tubes were incubated at 37° under a film of toluene for 4 days. Finally, excess inhibitor was added to the third pair of tubes which were also stored at 37° like the second pair. The total volumes of all tubes were equalized by addition of saline. After 4 days, solution of the precipitate by inhibitor was apparent in the third pair of tubes and the tubes were chilled to 4°. The precipitate was washed and estimated as before. There was good agreement between duplicates, and the results are summarized in Table 2.

Table 2. *Reversal of flocculation by addition of inhibitor to suspension of specific precipitate*

Wt. of egg albumin (mg. N)	Treatment of precipitate	Wt. of precipitate (mg. N)
0·07	16 hr. at 4°	0·53, 0·54
0·07	4 days at 37°	0·55, 0·53
0·07	Inhibitor added 4 days at 37°	0·36, 0·34

About 40% of the specific precipitate had been dissolved and it seemed likely that, if sterility could be maintained at 37° for 1 or 2 weeks, the precipitate would be completely dissolved.

DISCUSSION

The existence of non-flocculating antibodies whose presence can be demonstrated by their ability to inhibit the flocculation (or agglutination) caused by 'normal' antibodies has been demonstrated in horse antiovalbumin sera (Pappenheimer, 1940), human anti-*Rh* sera (Wiener, 1941) and in other cases. The experimental transformation of precipitating antibodies to a non-precipitating form has been effected by heat (cf. Kleczkowski, 1941), reaction with diazo compounds (Eagle, Smith & Vickers, 1936), formaldehyde (Eagle, 1938) and light (Tyler, 1945). In those cases which have been studied it appears that the inhibition produced by natural or artificial non-flocculating antibodies consists of a delay of flocculation, precipitation occurring eventually. Quantitative examination of horse antiovalbumin sera (Heidelberger, Treffers & Meyer, 1940) showed that when precipitation occurred in the presence of the inhibitory material, this substance was also carried down, as the total nitrogen precipitated was higher than when precipitation took place in its absence. It is, in fact, accepted that the 'univalent' or 'incomplete' antibodies are carried down when precipitation with complete antibody occurs in their presence. The inhibitor produced from antibody by the methods described in this paper is distinct from inhibitors previously described, since there is no evidence that it is attached to the antibody-antigen complex. Probably, for the same reason, the inhibition it produces seems to be complete.

On the basis of the lattice theory of precipitation reaction, hydrolysis would be expected to produce two inhibitors arising from two combining centres in different parts of the molecule. From the limited criteria available it appears that only one inhibitor, from the terminal quarter of the molecule, is produced by papain-HCN hydrolysis. This result would seem to be more in keeping with the occlusion theory of precipitation (Boyd, 1942), but it is possible that a second combining centre was destroyed during hydrolysis.

The properties of inhibitor suggest that it competes with antibody for the combining centre of antigen, both combinations being reversible, though in one case the precipitation which follows combination removes the reactants from solution. It is presumed that the specific combining centre present in the original antibody molecule is contained in the inhibitor fragment and it is clear that specificity is the property of only a fragment of the molecule. The active centre is contained in the terminal quarter of the molecule and from its rapid destruction after 72 hr. treatment with carboxypeptidase it is probably quite localized. In view of the general importance of protein specificity in biological reactions it is desirable that the minimum chain length of the active polypeptide should be established. In the case of the antigen silk fibrin (Landsteiner, 1942) it appeared that the specificity was determined by a sequence of only six or eight amino-acid residues, and in the adrenocorticotropic hormone, the specific activity survives peptic hydrolysis and partial acid hydrolysis (Li, 1947). In neither case, however, was the polypeptide responsible for the specific action isolated.

In this case, reduction of the inhibitor to its minimum size seems to depend on finding an aminopeptidase preparation of sufficient stability and width of specificity. As there is no evidence to suggest that the combining centre will be in the same position in different antibody molecules, the use of other antibodies may be preferable. The minimum size of the specificity determining polypeptide will probably differ in different antibodies, and will pre-

sumably depend upon how far the specific configuration is determined by amino-acid sequence and how far by the influence of other spacially adjacent sections of the molecule.

SUMMARY

1. The effect of a wide variety of hydrolytic conditions on the immunological activity of rabbit anti-ovalbumin has been examined.

2. Papain-hydrocyanic acid hydrolysis produces a quarter molecule unable to flocculate with the antigen, but able to inhibit flocculation of the complete system.

3. This inhibitory fragment appeared to have arisen from the terminal quarter of the molecule.

4. Attempts to reduce the size of the inhibitor further without destroying its activity were only partially successful.

5. A study of the interactions of antibody, inhibitor and antigen showed that complete inhibition of flocculation could be produced. Inhibition or flocculation could be reversed and the conclusion was reached that inhibitor was probably competing with antibody for the same combining centres of the antigen.

6. There was no evidence to suggest that the inhibitor was carried down by the specific precipitate when flocculation occurred in its presence.

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The Components of the Dihydrocozymase Oxidase System

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It has been known for many years that cozymase is necessary for the oxidation of a number of intermediary metabolites by tissues. The cozymase (Co I) is reduced by the metabolite, activated by a specific dehydrogenase, to dihydrocozymase (Co I H_2). There are two well known ways whereby the Co I H_2 may be oxidized to reform Co I, viz. (1), by the anaerobic reaction with a substrate (S): $S + \text{Co I } H_2 \rightarrow \text{Co I} + SH_2$, which requires a single enzyme, SH_2 dehydrogenase; and (2), by the aerobic reaction

$$\text{Co I } H_2 + \tfrac{1}{2}O_2 \rightarrow \text{Co I} + H_2O,$$

which requires a complex of enzymes, which we may call the Co I H_2 oxidase system. The present investigation, which is primarily concerned with the elucidation of the components of this Co I H_2 oxidase system, led to a study of a less well known third method for the oxidation of Co I H_2, viz. its anaerobic oxidation by fumarate: Co I H_2 + fumarate → Co I + succinate. This reaction was demonstrated indirectly by Dewan & Green (1937*a*), who found an oxido-reduction linking β-hydroxybutyrate, which is a typical cozymase-requiring dehydrogenase, and succinic dehydrogenase, which does not require cozymase. Its mechanism has not hitherto been studied. It is shown below that this is a slow reaction, and it is somewhat doubtful whether it is of great quantitative importance *in vivo*.

While it is widely, although not universally, accepted that the Co I H_2 oxidase system includes diaphorase or Co I H_2 dehydrogenase (Dewan & Green, 1937*b*, 1938; Adler, Euler & Hellström, 1938; Straub, 1939) and cytochrome oxidase, there is no agreement concerning the components of the cytochrome system involved. This uncertainty regarding the reaction between diaphorase and cytochrome oxidase constitutes, in fact, a serious gap in our knowledge of intracellular respiration. Dewan & Green (1937*b*, 1938) believed that cytochromes *a* and *b*, but definitely not *c*, were required. Lockhart & Potter (1941), on the other hand, obtained good evidence for the participation of *c*. Okuniki & Yakushiji (1940) thought that *b* and *c* were definitely required, while the role of *a* was uncertain. The present investigation shows that cytochromes *c*, *a* and a_3 (but not cytochrome *b*) and the BAL-sensitive factor previously found as a component of the succinic oxidase system (Slater, 1948, 1949*a*) are components of the Co I H_2 oxidase system. Some of the findings of the present investigation have been reported briefly (Slater, 1949*b*).

METHODS

Enzyme preparations

Heart-muscle preparation was prepared essentially by the method previously described (Keilin & Hartree, 1947; Slater, 1949*c*), with the exception that the precipitate collected by centrifugation after bringing the pH to 5·7 was washed by rubbing with ice-cold water and recentrifuging. This washing did not reduce the Co I H_2 oxidase activity. The precipitate was then suspended in an equal volume of 0·25 M-phosphate buffer, pH 7·3 (cf. 0·1 M previously used). The solution was kept at 4° and used within 4 days of preparation. The Co I H_2 oxidase system in this preparation is much less stable than the succinic oxidase system; after 6 days at 4°, the Co I H_2 oxidase activity was only one-half of its initial value, while the succinic oxidase system was only slightly affected.

Kidney preparation was prepared in the same way as previously (Slater, 1949*c*), except that 0·9% NaCl was used for washing instead of water (cf. Schneider, Claude & Hogeboom, 1948).

Cytochrome c was the product with 0·34% Fe, prepared by the method of Keilin & Hartree (1945).

Diaphorase was prepared from heart muscle by a method similar to that of Straub (1939).

Lactic dehydrogenase was a semi-purified preparation. Straub's (1940*a*) method was followed up to the first precipitation with $(NH_4)_2SO_4$. The precipitate thus obtained was dissolved in water and fractionated with $(NH_4)_2SO_4$, the fraction precipitating between 0·3 and 0·6 saturation being collected and dissolved in 0·3 saturated $(NH_4)_2SO_4$.

Liver extract. This was used as the source of glutamic dehydrogenase. Sheep or horse liver was cut into pieces with scissors and treated with 5 vol. of ice-cold 0·02 M-phosphate buffer, pH 7·3, in a Waring blender for 2 min. The mixture was centrifuged at 2000 rev./min. (1250 *g*) for 20 min. in an International centrifuge (size 1) with 250 ml. cups and the cloudy supernatant centrifuged at 12,000 rev./min. (13,000 *g*) in a Servall angle centrifuge (type SS1) for 30 min. The supernatant was dialysed against changes of distilled water for 18 hr. at 4°. A small precipitate was removed by centrifuging in the Servall centrifuge for 30 min. at 12,000 rev./min. This extract was used on the day of preparation.

Cozymase

Preparation. Cozymase, prepared from baker's yeast by an unpublished modification by Ochoa of Williamson & Green's (1940) method, was 33–34% pure. (This purity is not as high as is usually obtained by Ochoa's (Dr Ochoa, personal communication) method, probably because of the strain of yeast used.) The coenzyme II content, kindly determined by Dr C. Liébecq, by its catalytic activity in the oxidation of glucose-6-phosphate (in the presence of its dehydrogenase) by $K_3Fe(CN)_6$, was less than 0·2%.

Determination of purity. Cozymase preparation (10 mg.) was dissolved in 2 ml. of 1·3% (w/v) $NaHCO_3$ in a manometric flask containing 5 mg. of $Na_2S_2O_4$ in a dangling tube. The flask was attached to a manometer and the gas phase made 95% N_2–5% CO_2 (v/v). After tipping the dangling tube into the solution, the reaction was allowed to proceed at room temperature until the evolution of CO_2 ceased (3–5 hr.). This method of reduction is essentially that of Ohlmeyer (1938). The flask was then removed from the manometer, 3 ml. of 0·87% Na_2CO_3 added and the solution shaken with air for 5–10 min. A 0·2 ml. sample of this solution was added to 2·8 ml. of 0·1 M-phosphate buffer, pH 7·3, and the optical density at 340 mμ. determined in a 1 cm. cell in a Beckman spectrophotometer, using phosphate buffer in the blank cell. Call this reading D_1. Lactic dehydrogenase preparation (0·1 ml.) and 0·01 M-pyruvate (0·1 ml.) were then added to both cells and the optical density again measured. The reading rapidly declines (within 1–2 min.) to a reading which may either remain stationary or decline slowly. In the latter case, readings are taken against time and extrapolated to the time of adding the dehydrogenase and pyruvate. Call this reading D_2. Then percentage purity is 80 (D_1-D_2). The factor 80 was calculated from the weight of cozymase taken, the molecular weight of cozymase and the molecular extinction coefficient found by Horecker & Kornberg (1948). D_2, which was usually about 20% of D_1, was much greater than the optical density of the preparation at 340 mμ. before reduction. Thus, reduction with $Na_2S_2O_4$ produces substances other than Co I H_2 which absorb at 340 mμ.; these are possibly the reduced forms of other *N*-substituted nicotinamides present in the cozymase preparation. The presence of such substances means that methods of determining purity depending upon the increase of the optical density at 340 mμ. after reduction (or upon the CO_2 produced from the $NaHCO_3$ buffer by the H^+ liberated by the reduction) will tend to give high values.

Preparation of Co I H_2. Two methods of reduction of the cozymase were used.

(*a*) Sodium dithionite $Na_2S_2O_4$. The cozymase was reduced as described in the above paragraph and the solution obtained after aeration of the $NaHCO_3$–Na_2CO_3 solution used. LePage's (1947) much simpler method was found unsuitable for this investigation, since the Co I H_2 in such solutions was found to be appreciably autoxidizable (cf. Adler, Hellström & Euler, 1936).

(*b*) Enzymic. Cozymase preparation (40 mg.), Na L-glutamate (200 mg.) and 5% nicotinamide (1 ml.) were added to 0·05 M-Na_2HPO_4 (8·6 ml.) and the mixture adjusted to pH 8·3 with a few drops N-NaOH. Liver extract (0·4 ml.) was then added and the solution allowed to stand at room temperature until the reduction of cozymase, determined by adding 0·1 ml. samples to 2·9 ml. of 0·1 M-phosphate buffer and measuring the optical density at 340 mμ., was a maximum (2–3 hr.). The mixture was then heated to 80° for 5 min. and the precipitate removed by centrifugation. Usually, a control solution was prepared in exactly the same way as above, omitting the cozymase. The yield of Co I H_2 was only 60–75% that of Co I used. The remainder was either unreduced or was possibly destroyed by a pyrophosphatase of the type described by Kornberg & Lindberg (1948). Nicotinamide does not inhibit this enzyme.

Co I H_2 solutions prepared by both methods suffered only slight oxidation while being kept at 4° for 4 days.

Measurement of enzyme activities

Co I H_2 oxidase system. Co I H_2 solution (sufficient to give an optical density at 340 mμ. of about 0·5–0·6 when diluted to 3·0 ml.) was added to phosphate buffer, pH 7·3, in a Beckman 1 cm. cell, the total liquid volume being 2·8 ml. and the phosphate concentration, 0·125 M, except where otherwise stated. The 'blank' Beckman cell contained 2·8 ml. of the phosphate buffer. At zero time, 0·2 ml. of the enzyme preparation suitably diluted (usually 50-fold in the case of the heart-muscle preparation) in 0·125 M-phosphate buffer, pH 7·3, was added to each cell and the optical density at 340 mμ. measured at approximately 20 sec. intervals for about 4 min. The readings were plotted as shown in Fig. 1, and the slope of the straight line ($=v_{uncorr.}$) measured. The phosphate concentration had an important effect on the rate of oxidation, which was optimal at 0·125 M-phosphate in the case of heart-muscle preparation.

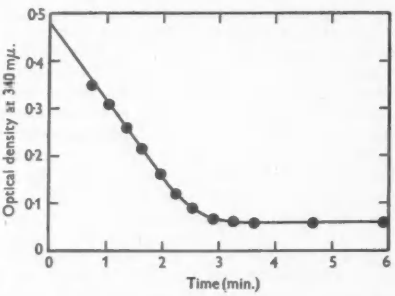


Fig. 1. Oxidation of Co I H_2 (initial concentration, $6·2 \times 10^{-5}$ M) by heart-muscle preparation (0·1 mg./ml.), added at zero time. Co I H_2 prepared by enzymic reduction. Temp. 22·8–23·2°. No correction for temperature.

The reaction is very sensitive to temperature, and the accuracy of this measurement is determined by the accuracy of the temperature correction. The temperature of the solution in the cell was measured immediately before adding the heart-muscle preparation and immediately at the end of the reaction. The rise of temperature was kept small by having the solution in the closed cell compartment of the Beckman for some time before commencing the measurement. The observed $v_{uncorr.}$ was converted to $v_{corr.}$ by the expression $\log_{10}(v_{corr.}/v_{uncorr.}) = 19{,}000\,(T_s - T)/2·303\,RT_sT$, where T is the mean observed absolute temperature, T_s is the absolute temperature chosen as the standard of the comparison (usually 293), R is the gas constant and 19,000 is the energy of activation of the reaction determined by measuring the rate of oxidation of the same preparation between 17·1 and 23·2°.

It was found that the same temperature coefficient governed the diaphorase determination (at least at low methylene blue concentrations) and it was assumed for the purposes of calculation, in the absence of any information, that it governed all the other oxidations of Co I H_2 considered in this paper. This assumption is certainly invalid in some cases, but any error caused by making it was minimized by keeping the temperature difference between different measurements in any one experiment as small as possible.

There was no need to aerate the solution during these measurements, since the amount of O_2 dissolved in water is about five times that necessary for complete oxidation of the Co I H_2, in the concentrations employed.

The heart-muscle preparation was diluted immediately before use, since its Co I H_2 oxidase was somewhat unstable in diluted solutions.

In the Figures which follow, all optical densities have been corrected to a fluid volume of 3·0 ml.

Succinic oxidase. This was determined as previously described (Slater, 1949*c*) except that no cytochrome *c* was added, the temperature was 25° and the diluted heart-muscle preparation was in the dangling tube, while the succinate was in the main solution. The temperature coefficient of this reaction was determined by measurements with the same heart-muscle preparation at 26·1 and 39·3°. The energy of activation, calculated from the rates at these two temperatures, was much less than that of the Co I H_2 oxidase system, viz. 7520 cal. This value, which was not affected by the addition of cytochrome *c*, is considerably different from those previously reported, viz. 11,200 by Hadidian & Hoagland (1939) and 11,500 by Thorn (1949). The value obtained in the present investigation was used only for comparing rates measured at room temperature with those at 38° and, until further measurements are made, too much significance should not be given to the actual value.

EXPERIMENTS

Components of the cytochrome system involved in the oxidation of dihydrocozymase-spectroscopic evidence

A fresh heart-muscle preparation was placed in the main compartment of a Thunberg tube and a solution of Co I H_2 (sufficient to give a final concentration of $3·3 \times 10^{-4}$ M when mixed with the heart-muscle preparation) in the hollow stopper. Oxygen was removed by evacuation, refilling with oxygen-free nitrogen, repeating the evacuation and refilling several times and finally evacuating. The Co I H_2 in the hollow stopper was then mixed with the heart-muscle preparation and the mixture immediately examined under a low-dispersion visual spectro-

scope. Strong *a* and *c* bands appeared immediately; a faint *b* band appeared on further standing. All bands disappeared immediately when the tube was opened to air and shaken. Essentially the same results were obtained whether the Co I H_2 was prepared by reduction with sodium dithionite or enzymically. No bands appeared when Co I replaced the Co I H_2.

When the same concentration of succinate was used instead of Co I H_2, there was the immediate appearance of a definite *b* band, corresponding to about half-complete reduction, in addition to strong *a* and *c* bands. This degree of reduction of cytochrome *b* by succinate is approximately that expected from the reported oxidation-reduction potentials (Ball, 1938) and the concentration of the cytochromes in heart-muscle preparation (Slater, 1949*c*). Since the oxidation-reduction potential of cozymase (−0·29 V. at pH 7·3, calculated from Borsook, 1940) is much less than that of the cytochromes, it would be expected, purely on thermodynamic grounds, that cytochrome *b* would be completely reduced by the concentration of Co I H_2 used. The finding that cytochrome *b* is only partially (and slowly) reduced by Co I H_2 under the conditions of this experiment might be because (1) cytochrome *b* is not on the main pathway for the oxidation of Co I H_2 by heart-muscle preparation, but is concerned in a side reaction; or (2) the oxidation of Co I H_2 by heart-muscle preparation is very slow compared with that of succinate. The second possibility can be definitely excluded since, as is shown below, the Co I H_2 oxidase activity of heart-muscle preparation is of the same order as its succinic oxidase activity, when both are measured in the presence of optimal substrate. With the concentrations used in this experiment, the Co I H_2 oxidase activity must have been considerably greater than the succinic oxidase.

When the above experiment with Co I H_2 was repeated with cyanide also present in the reaction mixture, the faint *b* band slowly increased in intensity, until it eventually corresponded to complete reduction.

When succinate (final concentration, $3{\cdot}3 \times 10^{-4}$ M) was added to heart-muscle preparation treated with BAL (under such conditions that the succinic oxidase system was completely inactivated) and the mixture shaken with air, a strong *b* band, but no *c* or *a* bands, was visible (cf. Slater, 1949*a*). If the same concentration of Co I H_2 was used, no bands were visible. It is shown below that the treatment with BAL inactivates the Co I H_2 oxidase system as well as the succinic oxidase system. In another experiment, heart-muscle preparation was shaken in air with BAL until all the BAL was oxidized. When this was freed from air no bands were visible (residual BAL will, under anaerobic conditions, reduce cytochromes *a*, *c* and *b*, the latter very slowly). On adding Co I H_2, the bands of *c* and *a* appeared very much more slowly than with untreated heart-muscle preparation.

Strong *a* and *c* bands, accompanied by a barely visible *b* band, were observed when lactate, lactic dehydrogenase, cozymase and cyanide were added to heart-muscle preparation, in the presence of air. If the lactic dehydrogenase was omitted, the bands still appeared, but much more slowly, probably due to the presence of a small amount of lactic dehydrogenase in the heart-muscle preparation (Keilin & Hartree, 1940). No bands appeared if the lactate, cozymase or cyanide were omitted. The cyanide serves a double purpose of enabling the reduction of the cozymase by the lactate-lactic dehydrogenase by removing the pyruvate formed (Green & Brosteaux, 1936), and of preventing the oxidation of the Co I H_2 through the cytochrome oxidase, which would rapidly exhaust the substrate. The *b* band was not reinforced by adding urethane, a procedure which strongly intensifies the *b* band when its reduction is, in the absence of urethane, slow compared with its oxidation.

It was found by the following experiments that the *a* band, formed by adding Co I H_2 to heart-muscle preparation, was due to both cytochromes *a* and a_3, as is the case when succinate is the reducing agent (Keilin & Hartree, 1939).

(1) When Co I H_2 was added to heart-muscle preparation under anaerobic conditions and the tube then filled with CO, the *a* band was broadened in the direction of shorter wavelength, due to the formation of cytochrome a_3–CO which absorbs at 589 mμ.

(2) Heart-muscle preparation was diluted tenfold with strong sucrose syrup. After removal of air, Co I H_2 was added and the solution examined with the visual spectroscope, using blue-violet light. The γ-band at 448 mμ., which Keilin & Hartree (1939) have shown to be due mainly to cytochrome a_3, was equally as strong as when succinate was the reducing agent. When CO was admitted, this band became very faint, while there was an intensification of the previously faint band at about 430 mμ., due to cytochrome *b*. This change of spectrum is due to the formation of a_3–CO which absorbs at 432 mμ., leaving the faint γ-band of cytochrome *a* at 452 mμ. unaffected (Keilin & Hartree, 1939).

(3) A very distinct band of a_3–CN at 590 mμ. was visible after standing when Co I H_2 was added to heart-muscle preparation in the presence of potassium cyanide, under anaerobic conditions. This band disappeared immediately on admitting air, while the *a* and *c* bands were unaffected.

The aerobic oxidation of Co I H_2 *by heart-muscle and kidney preparations*

The activity of the Co I H_2 oxidase system in heart-muscle preparation may be very conveniently studied by measuring the rate of decrease of the

optical density at 340 mμ., in a Beckman spectrophotometer. The actual details of the measurement are given in the Method section. The dilution of the heart-muscle preparation in this measurement was usually about 750-fold. At this dilution, the scattering of light by the insoluble heart-muscle preparation is not great and is eliminated from the measurements by adding the preparation to both the 'blank' and the 'test' cuvettes. An actual measurement is given in Fig. 1. It can be seen that a uniform rate of oxidation of Co I H_2 was maintained until nearly all the Co I H_2 was oxidized, indicating that the enzyme system must have a very high affinity for the Co I H_2, or that the first step of the reaction series does not limit the overall reaction rate, until nearly all the Co I H_2 is oxidized.

Curve 1, Fig. 2, shows that, under the conditions of the test, Co I H_2 was oxidized very slowly until the heart-muscle preparation was added (at the first arrow), when a rapid oxidation occurred. The optical density at 340 mμ. was not brought to zero by the heart-muscle preparation (see also Fig. 1). This is due to the presence of substances in the Co I H_2 preparation other than Co I H_2 which absorb at 340 mμ. The same final reading was obtained when the Co I H_2 was oxidized by lactic dehydrogenase and pyruvate ($3{\cdot}3 \times 10^{-4}$M) (curve 2, Fig. 2). Since the equilibrium constant of the reaction

$$\text{Pyruvate} + \text{Co I H}_2 \rightleftharpoons \text{Lactate} + \text{Co I}$$

is very high (Euler, Adler, Gunther & Hellström, 1937), the forward reaction proceeds virtually to completion under the conditions of this experiment.

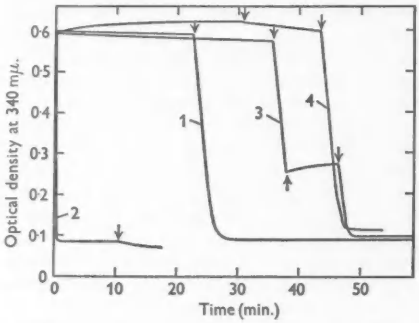


Fig. 2. Oxidation of Co I H_2 (prepared by enzymic reduction) by heart-muscle preparation (H.M.P.) and by lactic dehydrogenase and pyruvate. Final concentrations: Co I H_2, $8{\cdot}2 \times 10^{-5}$M (at commencement of reaction); H.M.P., 0·074 mg./ml.; pyruvate, $3{\cdot}3 \times 10^{-4}$M; lactic dehydrogenase (L.D.), one-thirtieth of concentrated preparation; methylene blue (M.B.), $6{\cdot}25 \times 10^{-5}$M; KCN, 0·01M; phosphate buffer, 0·125M. The 'blank' cuvette contained the appropriate amount of a solution prepared in the same way as the Co I H_2, without the addition of the cozymase. Temp. 27·7–31·0°. No correction for temperature.

Curve	Zero time	Added at 1st arrow	Added at 2nd arrow	Added at 3rd arrow
1	Co I H_2	H.M.P.	—	—
2	Co I H_2, L.D., pyruvate	M.B.	—	—
3	Co I H_2	H.M.P.	KCN	M.B.
4	Co I H_2, KCN	H.M.P.	M.B.	—

Since there is evidence (Adler *et al.* 1936; Hellström, 1937; Karrer & Benz, 1936) that the reduction of Co I by $Na_2S_2O_4$ (dithionite) to Co I H_2 proceeds through the semiquinone or half-reduced compound Co I H., it was necessary to consider the possibility that Co I H_2 is oxidized in two distinct steps:

$$(1)\quad \text{Co I H}_2 + \tfrac{1}{2}\text{O}_2 \rightarrow \text{Co I H.} + \tfrac{1}{2}\text{H}_2\text{O}$$
$$(2)\quad \text{Co I H.} + \tfrac{1}{2}\text{O}_2 \rightarrow \text{Co I} + \tfrac{1}{2}\text{H}_2\text{O}$$

Overall reaction:

$$(3)\quad \text{Co I H}_2 + \tfrac{1}{2}\text{O}_2 \rightarrow \text{Co I} + \text{H}_2\text{O}.$$

Adler *et al.* (1936) and Hellström (1937) have studied in detail the properties of a yellow substance which appears transiently when Co I is reduced by $Na_2S_2O_4$ in neutral solution, and is stabilized in alkaline solution. This yellow substance, which is believed by these authors to be Co I H., has an absoption maximum at 360 mμ. and shows a considerable absorption at 340 mμ. (amounting to at least 39 % that of Co I H_2). Thus, if any Co I H. accumulated during the oxidation of Co I H_2, the rate of decrease of the optical density might not be an accurate index of the rate of the overall reaction (3). The accumulation of Co I H. was tested experimentally by measuring the rate of the decline of the optical density at 340 and 380 mμ. with the same heart-muscle preparation. The ratio of the two rates (0·204) was practically the same as the relative optical densities of Co I H_2 at these two wavelengths (0·212). This shows that there was no accumulation of Co I H., since this substance absorbs more than Co I H_2 at 380 mμ. This was confirmed by following the optical density at 400 mμ. during the oxidation of Co I H_2. The latter absorbs very slightly at this wavelength, whereas the yellow substance absorbs strongly. Thus any accumulation of Co I H. would be revealed by an increase of the optical density at 400 mμ., but no such increase was found. It is concluded that the rate of decrease of the optical density at 340 mμ. is a reliable measure of the reaction (3) and enables the calculation of the Q_{O_2} (Co I H_2) of the enzyme preparations.

The finding that Co I H. does not accumulate during the course of the aerobic oxidation of Co I H_2 does not dismiss the possibility that the reaction proceeds in two single electron steps, since reaction (2) may be very much more rapid than reaction (1).

There are, in fact, good reasons for believing that the oxidation proceeds in single electron steps (Michaelis & Schubert, 1938; Luvalle & Goddard, 1948).

Effect of inhibitors of cytochrome oxidase. In curve 3, Fig. 2, cyanide (0·01 M finally) was added at the second arrow after about two-thirds of the Co I H_2 had been oxidized. This stopped the oxidation, which was restored by the addition of methylene blue at the third arrow. After the addition of cyanide, the optical density actually increased slightly. This is undoubtedly due to combination of oxidized cozymase (or other *N*-substituted nicotinamide compounds), either originally present in the Co I H_2 preparation or formed by the oxidation of the Co I H_2 by the heart-muscle preparation, with cyanide to give a compound which absorbs at 340 mμ. (Meyerhof, Ohlmeyer & Möhle, 1938). Co I H_2 does not react with cyanide.

In order to measure more accurately the degree of inhibition caused by the cyanide, the Co I H_2 and potassium cyanide were kept in contact for 30 min., until a stable reading was obtained (curve 4, Fig. 2). The heart-muscle preparation was then added at the first arrow. It can be seen that, in the presence of 0·01 M-cyanide, the reaction is brought almost completely to a standstill. More accurate measurements with more concentrated heart-muscle preparations showed that the inhibition by 0·01 M-cyanide was 99·6 %. Inhibitions by lower concentrations of cyanide are shown in Table 1.

The rate of oxidation of Co I H_2 by heart-muscle preparation in the presence of cyanide and methylene blue is a measure of the diaphorase activity of the heart-muscle preparation (p. 494).

Co I H_2, prepared as described in the Method section by reduction with $Na_2S_2O_4$, cannot be used for these experiments since, in the absence of any enzyme, the addition of potassium cyanide to such Co I H_2 preparations caused a rapid decrease of the optical density at 340 mμ. The reason for this is not known.

That the inhibition by cyanide is easily reversible was shown by the following experiment. Undiluted heart-muscle preparation was kept in contact with 10^{-3} M-cyanide for 30 min. at room temperature (*A*). It was then diluted 1500 times for measurement of Co I H_2 oxidase activity. A control (*B*) was treated in the same way as *A* without the addition of cyanide. Additional measurements were made in which cyanide was added to the diluted heart-muscle preparation *B* to final concentrations of 10^{-3} M and $6{\cdot}67 \times 10^{-7}$ M-potassium cyanide respectively, the latter being the concentration of cyanide after dilution of *A*. The results (Table 2) clearly show that the degree of inhibition is governed only by the concentration of cyanide during the measurement of the enzyme activity and not by the concentration during standing before dilution. It was found in another experiment that the degree of inhibition by 10^{-3} M-potassium cyanide was independent of the concentration of the heart-muscle preparation (over a 20-fold

Table 1. *Effect of inhibitors of cytochrome oxidase on coenzyme oxidase activity of heart-muscle preparation*

Inhibitor	Concentration (M)	Inhibition (%)
Cyanide	$6{\cdot}67 \times 10^{-7}$	11
	$3{\cdot}33 \times 10^{-4}$	93·9
	5×10^{-4}	96·9
	1×10^{-3}	98·3
	$3{\cdot}33 \times 10^{-3}$	98·9
	1×10^{-2}	99·6
Azide	1×10^{-3}	72·2
	5×10^{-3}	88·7
	1×10^{-2}	91·7
	2×10^{-2}	93·6
	5×10^{-2}	96·0
Hydroxylamine	$3{\cdot}33 \times 10^{-3}$	30
	1×10^{-2}	63·5
	$3{\cdot}33 \times 10^{-1}$	92
Sulphide	1×10^{-4}	96·3
	1×10^{-2}	99
Fluoride	1×10^{-2}	7
	3×10^{-2}	14

	CO (%)	O_2 (%)	
Carbon monoxide: H.M.P.* no. 1	80	20	37
H.M.P. no. 2†	79	21	11·6
H.M.P. no. 2†	95·1	4·9‡	52·4

* H.M.P. = heart-muscle preparation.
† These experiments were carried out in 0·256 M-phosphate, compared with 0·125 M-phosphate in all other experiments.
‡ The Co I H_2 oxidase activity in 95 % N_2, 5 % O_2 was the same as in air.

Table 2. *Reversibility of inhibition by cyanide of* Co I H_2 *oxidase*

(See text for details.)

Heart-muscle preparation	Concentration of KCN (M) During standing	Concentration of KCN (M) During measurement of Co I H_2 activity	Co I H_2 oxidase ($v_{corr.}$)	Inhibition (%)
B	0	0	0·0350	—
B	0	$6{\cdot}67 \times 10^{-7}$	0·0310	11
B	0	10^{-3}	0·00052	98·5
A	10^{-3}	$6{\cdot}67 \times 10^{-7}$	0·0311	11

range of concentrations). It follows from these two experiments that the inhibition by cyanide is completely reversible by dilution.

Table 1 shows that other inhibitors of cytochrome oxidase, viz. azide, hydroxylamine, sulphide and CO also inhibit the oxidation of Co I H_2. Fluoride, which has only a very slight effect on cytochrome oxidase (Borei, 1945), also had little effect on the Co I H_2 oxidation.

The effect of CO on the Co I H_2 oxidase systems of two heart-muscle preparations, which differed considerably in Co I H_2 oxidase activity, is shown in Table 1. The more active preparation (no. 1) was considerably more susceptible to inhibition by CO. The degree of inhibition by CO is usually expressed by the value of K, which equals $\frac{n}{1-n}\frac{[CO]}{[O_2]}$, where n = activity of system in presence of CO compared with that of control, and [CO] and [O_2] are the partial pressures of CO and O_2 in the gas mixture. The K of heart-muscle preparation no. 1 was 6·8 and that of no. 2 was 17·7.

The effect of bright light on the inhibition by CO is shown in Fig. 3. In the experiment shown in curve 3, the solution was alternately kept in the dark and placed in the bright light of a mercury vapour lamp. Whenever the solution was in the dark, the oxidation of the Co I H_2 followed the same course as the inhibited reaction kept in the dark throughout (curve 2), while whenever it was placed in the light, the curve is parallel to the uninhibited reaction (curve 1). Thus, bright light completely reverses the CO inhibition. Four successive reversals by light of the inhibition are shown. It is apparent that both the inactivation by CO and the reversal by light are very rapid processes. Curve 1, Fig. 3, shows that the light from the mercury vapour lamp had no effect on the rate of oxidation in air.

The easily reversible inhibition by low concentrations of cyanide, the light sensitivity of the CO inhibition and the fact that the relative susceptibility of the Co I H_2 oxidation to the inhibitors in Table 1 is the same as that of cytochrome oxidase leaves little doubt that cytochrome oxidase is concerned in the oxidation of Co I H_2. The Co I H_2 oxidase system in heart-muscle preparation would be convenient for the determination of the photochemical absorption spectrum of the CO-cytochrome oxidase compound.

Effect of BAL. It has been shown previously (Slater, 1948, 1949*a*) that treatment of heart-muscle preparation with 2:3-dimercaptopropanol (BAL) in air causes the destruction of a factor essential for the succinic oxidase system, acting between cytochromes *b* and *c*. Fig. 4 shows that treatment of heart-muscle

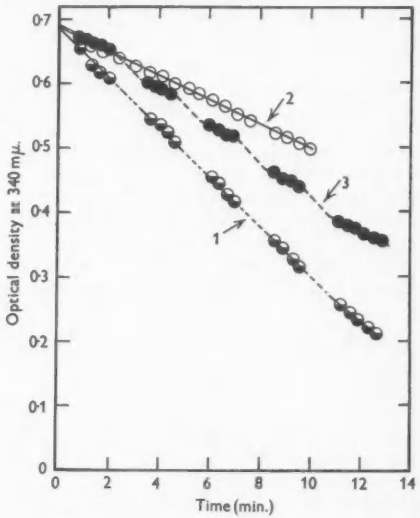


Fig. 3. Inhibition of Co I H_2 oxidase by CO and reversal by light. Co I H_2 prepared by enzymic reduction. Final concentrations, H.M.P., 0·029 mg./ml.; Co I H_2, $4{\cdot}8 \times 10^{-5}$M (at commencement of reaction); phosphate buffer, 0·256M. The reactions were carried out in 2 cm. cells with a narrow neck and close-fitting stopper. When filled (total vol. = 5·0 ml.), there was only a small gas space. The gas mixture was bubbled through the solution of Co I H_2 in phosphate buffer in the cell for some time. At zero time, the H.M.P. was added, the cell transferred to the Beckman compartment and readings taken with time. The effect of light was studied by removing from the Beckman and placing in the light from a mercury vapour lamp focused by two lenses for 1 min. periods. Periods when the cells were in the dark are shown by full lines on the graph, those in the light by dotted lines. Gas phases: curve 1, air; curves 2 and 3, 95·1% CO, 4·9% O_2. The measurements which were made between 22·2 and 22·9° have not been corrected for temperature.

preparation with 0·02 M-BAL for 15 min. at 39° inactivates the Co I H_2 oxidase system by 99 % (cf. curves 2 and 3), without affecting the diaphorase activity, measured by adding cyanide and methylene blue (curves 3, 4 and 5).

A comparison of curve 1, obtained with untreated heart-muscle preparation and curve 2, which serves as a control for the BAL treatment, shows that treatment of the heart muscle for 15 min. at 39° in the absence of BAL has some effect on the Co I H_2 oxidase activity. After such treatment, there is some lag in reaching the maximal rate of oxidation after

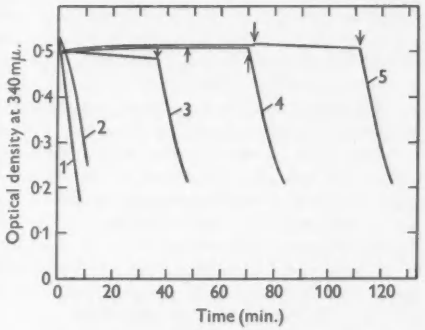


Fig. 4. Effect of treatment with BAL on Co I H_2 oxidase and diaphorase activity of heart-muscle preparation (H.M.P.). Co I H_2 prepared by enzymic reduction. H.M.P. 1, untreated. H.M.P. 2, shaken in air at 39° for 15 min., then diluted. H.M.P. 3, shaken with 0·02 M-BAL in air at 39° for 15 min., then diluted ('General procedure' of Slater, 1949*a*). Final concentrations: H.M.P., 0·037 mg./ml.; methylene blue (M.B.), $6{\cdot}25 \times 10^{-5}$ M; KCN, 0·01 M; phosphate buffer, 0·125 M. The 'blank' cuvette contained the appropriate amount of a solution prepared in the same way as the Co I H_2, without the addition of the cozymase. Measurements were carried out at temperatures between 25·5 and 30·2° and the curves corrected to 20° by appropriate adjustment of the time scale.

Curve	Added at: Zero time	1st arrow	2nd arrow
1	Co I H_2, H.M.P. 1	—	—
2	Co I H_2, H.M.P. 2	—	—
3	Co I H_2, H.M.P. 3	M.B.	—
4	Co I H_2, KCN	H.M.P. 3	M.B.
5	Co I H_2, KCN	H.M.P. 2	M.B.

adding the heart-muscle preparation to the Co I H_2. The maximal rate is, however, usually only about 10 % less than that given by an untreated heart-muscle preparation (in Fig. 4, it has been affected to a somewhat greater degree). The lag was much more pronounced if the warming to 39° was carried out at low phosphate concentrations. Shaking the heart-muscle preparation in air at 19° for 1 hr. or at 25° for 45 min. had no effect on the Co I H_2 oxidase activity. Treatment with BAL under these conditions had the same effect on the Co I H_2 oxidase system as for 15 min. at 39°.

For these experiments, Co I H_2, prepared by $Na_2S_2O_4$ reduction, was not as satisfactory as that prepared by enzymic reduction, since BAL treatment of heart-muscle preparation did not completely stop the oxidation measured by the decline of the optical density at 340 mμ. The residual oxidation amounted to 5–20 % of the initial rate. This is very likely due to bisulphite derived from the $Na_2S_2O_4$ (Adler *et al.* 1936; Hellström, 1937) in these Co I H_2 preparations, since Co I H_2 prepared enzymically behaved in the same way when sodium bisulphite ($6{\cdot}4 \times 10^{-5}$ M) was added.

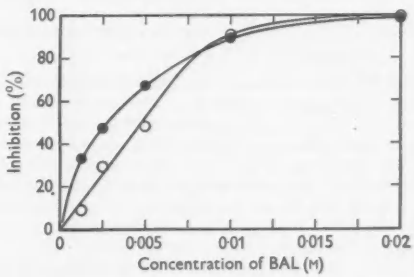


Fig. 5. Comparison of the inactivation of the succinic oxidase system and of the Co I H_2 oxidase system in heart-muscle preparation by treatment with different concentrations of BAL. 1 ml. of heart-muscle preparation (40 mg./ml.) treated with various concentrations of BAL in a total volume of 2 ml. for 45 min. at 25·7° with shaking in air. Concentration of phosphate buffer, 0·1 M. Heart-muscle preparation diluted to 0·48 mg./ml. for measurement of succinic oxidase activity and to 0·053 mg./ml. for measurement of Co I H_2 oxidase activity. Succinic oxidase activity measured at 25·9°. Co I H_2 oxidase activity measured at temperatures between 24·2 and 27·4° and corrected to same temperature for calculation of percentage inhibition. Q_{O_2} (μl. of O_2/mg. of fat-free dry wt./hr.) of controls at 25·9°; succinic oxidase, 291; Co I H_2 oxidase, 305; ●—●—, Co I H_2; ○—○—, succinic oxidase.

In Fig. 5, the effect of different concentrations of BAL on the Co I H_2 and succinic oxidase systems in heart muscle are compared. The two systems require approximately the same concentration of BAL for complete or nearly complete inactivation. The Co I H_2 oxidase system is appreciably more sensitive than the succinic oxidase to low concentrations of BAL.

Two possible explanations of the effect of BAL on the Co I H_2 oxidase system were considered: (1) the BAL-sensitive component of the succinic oxidase system is also a component of the Co I H_2 oxidase system; (2) coincident with the destruction of the BAL-sensitive component, there is an alteration of

the structure of the particles of the heart-muscle preparation which enables the diaphorase to dissociate from the particles so that much of the diaphorase is in solution and no longer readily accessible to the other components of the system. Although the fact that the Co I H_2 oxidase system was rather more susceptible than the succinic oxidase system to BAL made it unlikely, it was necessary to consider this second possibility, since it is known that diaphorase is more readily dissociated from the insoluble particles than are other components. If this second explanation is correct, it should be possible to reactivate the system after treatment with BAL by adding a large excess of purified diaphorase. Experiments along these lines are summarized in Table 3.

Table 3. *Effect of diaphorase and cytochrome* c *on the inactivation of* Co I H_2 *oxidase by BAL*

(*Exp.* 1. BAL treatment consisted of shaking concentrated heart-muscle preparation with 0·029 M-BAL for 80 min. at 15°. Co I H_2 oxidase activity of 1500-fold diluted heart-muscle preparation (0·023 mg./ml.) measured in 0·13 M-phosphate. Diaphorase added equalled 13·5 times that present in the heart-muscle preparation. Activity after addition of diaphorase corrected for rate of oxidation with diaphorase alone (viz. 0·0127). Co I H_2 prepared by $Na_2S_2O_4$ reduction.

Exp. 2. BAL treatment consisted of shaking concentrated heart-muscle preparation with 0·0025 M-BAL for 45 min. at 25°. Co I H_2 oxidase activity of 375-fold diluted heart-muscle preparation (0·093 mg./ml.) measured in 0·022 M-phosphate. Diaphorase added equalled eight times that present in the heart-muscle preparation. Added cytochrome *c*, $3{\cdot}8 \times 10^{-5}$ M. Activity after addition of diaphorase corrected for rate of oxidation with diaphorase alone (0·0033). Co I H_2 prepared by enzymic reduction.)

	Co I H_2 oxidase activity ($v_{corr.}$)		
Exp. 1	Control	BAL treated	Inactivation (%)
No addition	0·0208	0·0046	78
+diaphorase	0·0188	0·0049	73
Exp. 2			
No addition	0·0298	0·0163	45·1
+diaphorase	0·0532	0·0288	45·9
+cytochrome *c*	0·0340	0·0186	45·3
+diaphorase +cytochrome *c*	0·0557	0·0308	44·7

It was necessary to limit the amount of diaphorase added, since isolated diaphorase is appreciably autoxidizable, and oxidizes Co I H_2 without any other addition. Measurements were corrected for this 'blank' oxidation. In Exp. 1, which was carried out in 0·13 M-phosphate buffer, the diaphorase activity of the added diaphorase solution was 13·5 times that of the heart-muscle preparation. In this experiment, added diaphorase had little effect on either the control or BAL treated heart-muscle preparation.

The Co I H_2 oxidase system is much less active at low phosphate concentrations than in 0·13 M-phosphate. Exp. 2 shows the effect of diaphorase on the inactivation caused by BAL at 0·022 M-phosphate. In this experiment, cytochrome *c* ($3{\cdot}8 \times 10^{-5}$ M) was also added since it was possible that, at this low phosphate concentration, the accessibility of the cytochrome *c* and not of the diaphorase to the rest of the system was limiting the activity of the Co I H_2 oxidase (cf. the effect of cytochrome *c* on the activity of the succinic oxidase system at low phosphate concentrations, Slater, 1949*d*). In this case, eight times as much diaphorase as present in the heart-muscle preparation was added (the actual amount added was greater than in Exp. 1, but more heart-muscle preparation was used). It is apparent that, although diaphorase caused a considerable increase in activity of the control at the low phosphate concentration used in this experiment, it did not have any effect on the inactivation caused by the BAL. Similarly, cytochrome *c* had little effect on the inactivation; it also has no effect on the inactivation caused by BAL of the succinic oxidase system (Slater, 1949*a*).

The experiments summarized in Table 3 make it very unlikely that BAL acts on the Co I H_2 system by causing the dissociation of diaphorase from the particles of the heart-muscle preparation.

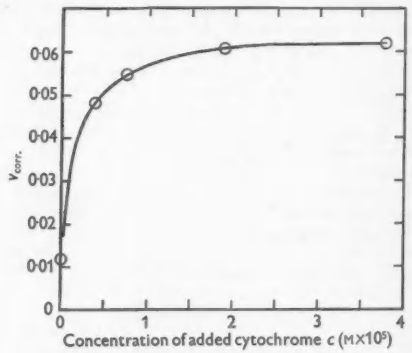


Fig. 6. Effect of added cytochrome *c* on Co I H_2 oxidase activity of kidney preparation. Final concentrations, kidney preparation, 0·165 mg./ml.; phosphate buffer, 0·045 M. Reactions carried out between 19·6 and 22·8°; rates corrected to 20°.

The Co I H_2 oxidase system behaved in the same way as the succinic oxidase system in the following respects: (1) treatment of the heart-muscle preparation with BAL in the absence of air had a negligible effect on the enzyme activities; (2) the effect of the BAL was not due just to the hydrogen peroxide produced during the oxidation of BAL, since hydrogen peroxide formed by notatin-glucose had a much smaller effect on the system.

Effect of added cytochrome c. Added cytochrome *c* did not have a very marked effect on the Co I H_2 oxidase activity of heart-muscle preparation, even at low phosphate concentrations, when added cytochrome *c* has its maximal effect on the succinic oxidase system (Slater, 1949*d*). A very marked effect of added cytochrome *c* was, however, obtained with kidney preparation (Fig. 6), $3{\cdot}8 \times 10^{-5}$M-cytochrome *c* being necessary for the maximum effect. The succinic oxidase system in kidney is likewise strongly stimulated by the addition of cytochrome *c* (Keilin & Hartree, 1940, 1945, 1949*a*; Slater, 1949*c*).

The anaerobic oxidation of Co I H_2 *by fumarate*

Neither fumarate (0·027 M) nor malonate (0·0133 M) had any effect on the aerobic oxidation of Co I H_2 by heart-muscle preparation.

However, the finding that the BAL-sensitive factor was common to both the succinic oxidase and Co I H_2 oxidase systems and that, although cytochrome *b* was not in the main pathway for the oxidation of Co I H_2, a faint *b* band appeared when Co I H_2 was added to heart-muscle preparation suggested some link between the two oxidase systems. If there is such a link between the two systems, one would expect that the more positive fumarate would be able to oxidize the very negative Co I H_2. Indeed, that such a reaction does occur was suggested by Dewan & Green (1937*a*) who found that, under anaerobic conditions, fumarate brings about the oxidation of β-hydroxybutyrate and that cozymase is required for this reaction. A direct observation of the oxidation of Co I H_2 by fumarate has not, however, hitherto been reported in the literature. Accordingly, the effect of fumarate on Co I H_2 and heart-muscle preparation under anaerobic conditions was investigated.

Fig. 7, curve 1, shows that when Co I H_2 was added to heart-muscle preparation in the absence of air, no oxidation of the Co I H_2 occurred. As soon as fumarate was added, however, the Co I H_2 was oxidized.

This oxidation of Co I H_2 by fumarate could also be studied in the presence of air by adding cyanide to inhibit almost completely the aerobic oxidation of the Co I H_2 and then adding fumarate (Fig. 8). The slow oxidation in the presence of cyanide alone was subtracted from the rate obtained after adding fumarate in order to obtain the rate due to fumarate alone.

Reduction of cozymase by malic dehydrogenase and malate formed from the fumarate by the action of fumarase was insignificant. The thoroughly washed heart-muscle preparation probably contains very little malic dehydrogenase.

The following conclusions may be drawn from the experiments summarized in Figs. 7 and 8 and from other experiments:

(1) When the aerobic oxidation of Co I H_2 is prevented either by removing oxygen or by adding cyanide, the Co I H_2 may be oxidized by fumarate.

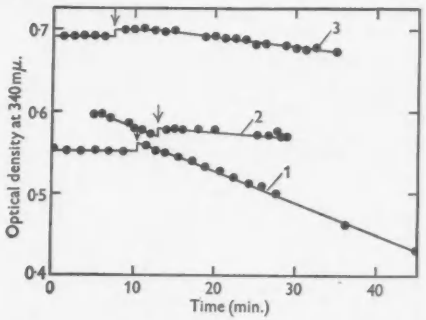


Fig. 7. Anaerobic oxidation of Co I H_2 by fumarate. These experiments were carried out in a Thunberg tube with optically plane faces. Light path = 0·5 cm. There was no blank cuvette; thus the measurements of optical density included light absorbed and scattered by the Thunberg vessel, by the heart-muscle preparation and the other additions as well as by the Co I H_2. The tubes with contents shown in second column below were evacuated and contents of hollow stopper added at arrow. Final concentrations: heart-muscle preparation, 0·46 mg./ml.; phosphate buffer, 0·13 M; Co I H_2 (prepared by $Na_2S_2O_4$ reduction), 6×10^{-5} M at commencement of reaction; fumarate, 0·027 M; malonate, 0·054 M. BAL treatment consisted of shaking concentrated heart-muscle preparation with 0·029 M-BAL for 80 min. at 15°. Measurements were carried out at temperatures between 20 and 25° and corrected to 20° by appropriate adjustment of time scale.

Curve	In main compartment	In hollow stopper
1	Co I H_2, H.M.P.	Fumarate
2	Co I H_2, H.M.P., fumarate	Malonate
3	Co I H_2, BAL-treated H.M.P.	Fumarate

(2) This anaerobic oxidation of Co I H_2 by fumarate is very slow compared with the aerobic oxidation. The mean rate of oxidation by fumarate in the presence of cyanide was found to be 2% that of the oxidation of Co I H_2 or of succinate at the temperature of the experiments (23·9–27·6°).

(3) Although the reaction is slow, the anaerobic oxidation of Co I H_2 by fumarate proceeds to completion. Thus the final value in curve 3, Fig. 8, was not decreased by adding pyruvate and lactic dehydrogenase. This final value is slightly higher than that obtained by aerobic oxidation (curve 1), probably because of the reaction between cyanide and cozymase (p. 489).

(4) The anaerobic oxidation was inhibited 83 % and the oxidation in the presence of cyanide 96 % by the addition of malonate in double the concentration of fumarate. This indicates that succinic dehydrogenase is concerned in the reaction.

(5) Treatment of the heart-muscle preparation with BAL inactivates the anaerobic oxidation by 60 %. The oxidation still remaining after the treatment with BAL was also strongly inhibited by malonate.

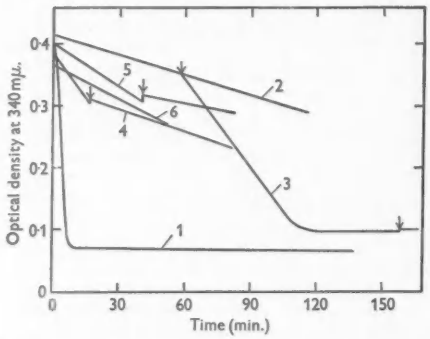


Fig. 8. Oxidation of Co I H_2 in presence of heart-muscle preparation (H.M.P.) and cyanide by fumarate. Co I H_2 prepared by enzymic reduction. H.M.P. 1, shaken in air at 39° for 15 min., then diluted. H.M.P. 2, shaken with 0·02 M-BAL in air at 39° for 15 min., then diluted. Final concentrations: H.M.P., 0·185 mg./ml.; Co I H_2, $5{\cdot}6 \times 10^{-5}$ M at commencement of experiment; KCN, 0·01 M; fumarate, 0·01 M; malonate, 0·02 M; pyruvate, $3{\cdot}3 \times 10^{-4}$ M; lactic dehydrogenase (L.D.), one-thirtieth of concentrated preparation; phosphate buffer, 0·125 M. The 'blank' cuvette contained the appropriate amount of a solution prepared in the same way as the Co I H_2, without the addition of the cozymase. Measurements were carried out at temperatures between 24·4 and 28·8° and corrected to 20° by appropriate adjustment of the time scale.*

The role of cytochrome *b* in this anaerobic reaction could be seen with the visual spectroscope. The faint *b* band obtained when Co I H_2 was added to heart-muscle preparation in the absence of air disappeared immediately on the subsequent addition of fumarate.

Diaphorase activity of heart-muscle preparation

If it is assumed that diaphorase is the first member of the respiratory chain with which Co I H_2 reacts (p. 495), the aerobic oxidation of Co I H_2 may be described by two reactions (each of which is probably the sum of a number of reactions), viz.

$$(1)\quad \text{Co I } H_2 + D \rightarrow \text{Co I} + DH_2,$$
$$(2)\quad DH_2 + \tfrac{1}{2}O_2 \rightarrow D + H_2O,$$

where D and DH_2 are oxidized and reduced diaphorase respectively. The rate of reaction (1) can be determined independently, since DH_2 is oxidized by methylene blue (MB)

$$(3)\quad DH_2 + MB \rightarrow D + MBH_2.$$

Thus the oxidation of Co I H_2 by methylene blue, in the presence of cyanide to stop reaction (2), is due to reaction (1), followed by reaction (3), followed by the reoxidation of methylene blue. Under the conditions of the experiments, this latter reaction was rapid (as shown by following the reaction at 650 mμ., at which wavelength methylene blue absorbs strongly). If reaction (3) is made very fast compared with reaction (1), the overall reaction will be a measure of reaction (1). It would be expected that reaction (3) could be made very rapid by increasing the methylene blue concentration sufficiently. However, it was found that the overall reaction continued to increase with increasing methylene blue concentration at concentrations of the latter which strongly absorbed at 340 mμ. and which oxidized Co I H_2 appreciably in the absence of any enzyme. This difficulty was overcome by extrapolating rates obtained at different methylene blue concentrations (corrected for the oxidation in the absence of enzyme) to infinite methylene blue concentration by the method of Lineweaver & Burk (1934) (cf. Slater, 1949*e*), since it was found that the relation activity: methylene blue concentration was a rectangular hyperbola. This extrapolated velocity must represent the rate of reaction (1). In the case of heart-muscle preparation this was found to be 3–4 times the Co I H_2 oxidase activity, measured in the absence of added cytochrome *c*. With the kidney preparation, the rate of reaction (1) was 2·2 times that of the aerobic oxidation of Co I H_2, in the presence of excess cytochrome *c*.

DISCUSSION

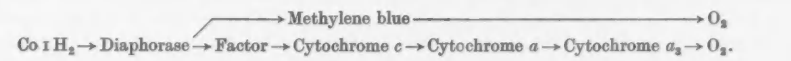
Components of the dihydrocozymase oxidase system

On the basis of the above experiments, it is suggested that the Co I H_2 oxidase system consists of the

* Added at

Curve	(Zero–30) (min.)	Zero time	1st arrow	2nd arrow
1	—	Co I H_2, H.M.P. 1	—	—
2	Co I H_2, KCN	H.M.P. 1	—	—
3	Co I H_2, KCN	H.M.P. 1	Fumarate	L.D., pyruvate
4	Co I H_2, KCN	H.M.P. 1, fumarate	Malonate	—
5	Co I H_2, KCN	H.M.P. 2, fumarate	Malonate	—
6	—	Co I H_2, H.M.P. 2	—	—

following components, the arrows denoting the pathway taken by the hydrogen atoms or electrons on their way from substrate to oxygen.



The addition of methylene blue enables the reaction to by-pass all the components except diaphorase. The factor is the BAL-sensitive component of the system and is probably the same as the factor previously found necessary for the oxidation of succinate (Slater, 1948, 1949*a*). This proposed scheme does not, of course, exclude the possibility that further components, not yet identified, may also be required (cf. Slater, 1949*a*). In particular, the possible role of the substance responsible for the *c* band (Keilin & Hartree, 1949*b*) of cytochrome has not yet been investigated.

We shall now consider, in turn, the evidence for the participation of each of these components.

Diaphorase. The finding that there exists in tissues an enzyme which acts as a carrier between Co I H_2 and methylene blue (Dewan & Green, 1937*b*, 1938; Adler *et al.* 1938; Straub, 1939) strongly suggested, by analogy with other dehydrogenases, that diaphorase acts *in vivo* by acting as a carrier between Co I H_2 and those members of the respiratory chain with a higher potential. Lockhart & Potter (1941) considered, on the other hand, that 'the participation of diaphorase in the cytochrome reduction remains to be shown, since its mere presence in the complete system is not enough to warrant the conclusion that it is an essential link in the transport mechanism between Co I H_2 and cytochrome *c*'. However, since diaphorase mediates the oxidation of Co I H_2 by methylene blue, it is presumably reduced by Co I H_2. The objection of Lockhart & Potter (1941) implies then that reduced diaphorase might be oxidized *in vivo* by a mechanism not involving the cytochrome system. Since the aerobic oxidation of Co I H_2 is completely sensitive to cyanide or to treatment with BAL, this hypothetical mechanism cannot be involved in the aerobic oxidation, and the function of diaphorase must be in some anaerobic oxido-reduction, which should be sufficiently rapid to account for the high diaphorase activity of various tissues. Since no such reaction has been demonstrated, it seems more likely that the function of diaphorase is in the aerobic oxidation of Co I H_2. This is supported by the following findings in the present investigation: (1) The rate of reduction of diaphorase in heart-muscle and kidney preparations by Co I H_2, calculated as described above, is sufficient to account for the very rapid aerobic oxidation of Co I H_2. (2) The Co I H_2 oxidase activity of heart-muscle preparation was greatly reduced at low phosphate concentrations, probably due to an impaired mutual accessibility of the components of the Co I H_2 oxidase system on the particles of the enzyme preparation. At these low phosphate concentrations, the system was considerably reactivated by the addition of a highly purified diaphorase solution. This reactivation resembles that produced by cytochrome *c* on the succinic oxidase system at low phosphate concentrations (Slater, 1949*d*) and strongly suggests that diaphorase is a component of the Co I H_2 oxidase system. However, the possibility that the diaphorase solution is, in this experiment, acting like the indifferent proteins discussed by Keilin & Hartree (1949*a*) cannot be excluded.

Factor. The conclusion that the BAL-sensitive factor previously described is a component of the Co I H_2 oxidase system, acting between diaphorase and cytochrome *c*, is supported by the following findings. (1) The fact that isolated diaphorase does not reduce cytochrome *c* (Lockart & Potter, 1941, confirmed in the present investigation) is suggestive, although not conclusive, evidence that an additional catalyst is required *in vivo*. (2) Treatment of a heart-muscle preparation with BAL, in the presence of air, inactivated the Co I H_2 oxidase system by 99%, without affecting the diaphorase activity, and the system was not reactivated by the addition of diaphorase or cytochrome *c*. The cytochrome *c* oxidase activity of heart-muscle preparation is not affected by this treatment (Slater, 1949*a*). (3) The rate of reduction of added cytochrome *c* by Co I H_2 in the presence of heart-muscle preparation is almost completely inhibited by the BAL treatment (Slater, 1950). Similarly, the rate of reduction of endogenous cytochrome *c* of heart-muscle preparation by Co I H_2 was greatly reduced by the BAL treatment. (4) The demonstration of the anaerobic oxidation of Co I H_2 by fumarate and the finding that this reaction is considerably inactivated by BAL indicates that the BAL-sensitive factor must act as a link between the succinic and Co I H_2 oxidase systems. This reaction is further discussed on p. 496.

Cytochrome c. There is a considerable body of evidence in the literature implicating cytochrome *c* in the oxidation of Co I H_2. That added cytochrome *c* is reduced by Co I H_2 in the presence of tissue preparations was shown by Adler *et al.* (1938), Hawthorne & Harrison (1939), Straub (1940*b*), Lockhart & Potter (1941). The catalytic effect of added cytochrome *c* on the oxidation of Co I H_2 or of those substrates which require cozymase for their oxidation was reported by Hawthorne & Harrison (1939), Straub (1940*b*), Lockhart & Potter (1941) and Okuniki & Yakushiji (1940).

The present work supports these conclusions and adds the further evidence that the endogenous cytochrome *c* in the heart-muscle preparation is very rapidly reduced by the Co I H_2. It was considered important to investigate this point since some substances, e.g. ascorbic acid, rapidly reduce cytochrome *c* in solution, but not the endogenous cytochrome *c* of the heart-muscle preparation.

The finding, in the present investigation, that Co I H_2, reduced either by sodium dithionite or by a dehydrogenase system, rapidly reduces the cytochrome *c* in the heart-muscle preparation does not agree with Dewan & Green (1938), who observed a reduction of cytochromes *b* and *a*, but not of *c* (cf. also Green, 1936). The explanation of this difference between the results of Green and the present investigation is not clear.

Dewan & Green (1938) did not obtain any stimulation of the oxidation of triosephosphate, lactate or malate by the addition of cytochrome *c*. This is understandable, since it is probable that the enzyme preparation contained sufficient cytochrome *c* not to limit the rate of oxidation. Hawthorne & Harrison (1939) found a stimulation by cytochrome *c* only at low enzyme concentrations. In the present study, cytochrome *c* had relatively little effect on the Co I H_2 oxidase activity of heart-muscle preparation even at low phosphate concentrations, where it was found to have its maximal effect in the succinic oxidase system (Slater, 1949*d*). However, it was strongly active in stimulating the Co I H_2 oxidase of kidney preparation, increasing the activity by 11-fold in one experiment.

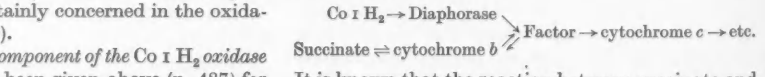
Cytochromes a *and* a_3. The observation made by Dewan & Green (1938) and in the present investigation that cytochrome *a* is rapidly reduced by Co I H_2 and rapidly oxidized by air is strong evidence that cytochrome *a* is concerned in the reaction. That it acts after cytochrome *c*, as postulated, is less certain, but this is the most likely possibility, since cytochrome *a* has an oxidation-reduction potential 0·03V. higher than cytochrome *c* (Ball, 1938) and it is closely associated with cytochrome a_3 (Keilin & Hartree, 1939). It is not possible, however, to exclude the possibility that the positions of *a* and *c* are interchanged or even that *a* and a_3 are on alternative pathways for the passage of hydrogen atoms or electrons from one of the members of the respiratory chain with lower potential to oxygen.

It was shown that the *a* band was double, being composed both of cytochrome *a* and a_3. Keilin & Hartree (1939) have shown that cytochrome a_3 is probably identical with cytochrome oxidase. Cytochrome oxidase is certainly concerned in the oxidation of Co I H_2 (p. 490).

Cytochrome b *not a component of the* Co I H_2 *oxidase system.* Reasons have been given above (p. 487) for believing, in opposition to Dewan & Green (1938), that cytochrome *b* is not on the main pathway for the oxidation of Co I H_2. This is supported by the slowness of the oxidation of Co I H_2 by fumarate; if cytochrome *b* were rapidly reduced by Co I H_2, the latter would be rapidly oxidized by fumarate, since the oxidation of reduced cytochrome *b* by fumarate is a fast reaction. Hawthorne & Harrison (1939) agreed with Dewan & Green (1938) that the oxidation of Co I H_2 by tissues normally proceeded through cytochromes *a* and *b*, without involving cytochrome *c*, since they obtained a rapid oxidation by a preparation which they consider was freed from cytochrome *c* by repeated *iso*electric precipitation. It is, however, very doubtful if the cytochrome *c* was removed by this procedure. Okuniki & Yakushiji (1940) also believe that cytochrome *b* is required, but their evidence is not at all convincing. They found a slight stimulation of the oxygen uptake when Yakushiji & Mori's (1937) preparation of cytochrome *b* was added to an incomplete system, but it is not generally accepted that Yakushiji & Mori's (1937) preparation actually contains any cytochrome *b* (Keilin & Hartree, 1939). Potter & Lockhart (1939) and Lockhart & Potter (1941) found that a heart-muscle preparation obtained by precipitation at pH 4·6 did not catalyse the reduction of cytochrome *c* by Co I H_2, although it reduced methylene blue, suggesting that some factor operated between diaphorase and cytochrome *c* and thought that this might be cytochrome *b*. However, such acid treatment affects the physical properties of heart-muscle preparation (Keilin & Hartree, 1940, 1949*a*) and this physical alteration would be quite sufficient to explain the inactivation. It is unlikely that cytochrome *b* is affected by this treatment since the rate of reduction of methylene blue by succinate is not greatly inhibited (Keilin & Hartree, 1940) and there is evidence that cytochrome *b* is concerned in this reaction (Ball, Anfinsen & Cooper, 1947; Slater, 1949*c*).

The fact that cytochrome *b* is not concerned in the oxidation of Co I H_2 draws attention to the specific association of cytochrome *b* with the oxidation of succinate and increases the evidence that succinic dehydrogenase and cytochrome *b* are identical (cf. Slater, 1949*c*). This evidence is, in the case of animal tissues, all of a negative nature, but Pappenheimer & Hendee's (1949) recent studies with diphtherial cytochrome *b* strongly suggest that the two are identical in this organism.

Anaerobic oxidation by fumarate. The Co I H_2 and succinic oxidase systems may be described in the one scheme as follows:



It is known that the reaction between succinate and cytochrome *b* is easily reversible. If the reaction

between cytochrome *b* and the factor is also reversible to a certain extent, the slow reduction of cytochrome *b* by Co I H_2 and the anaerobic oxidation of Co I H_2 by fumarate are understandable, since in the absence of oxygen and the presence of fumarate, the reaction

Co I H_2 → Diaphorase → Factor
→ cytochrome *b* → Fumarate

can take place.

The inhibition of this reaction by malonate, which affects the reaction between cytochrome *b* and fumarate, and by BAL treatment, which destroys the factor, is in agreement with this series of reactions. The fact that inactivation by BAL treatment is not complete suggests, however, that the oxidation of Co I H_2 by fumarate proceeds not only by the reaction shown, but also by a BAL-insensitive pathway. The rate of this BAL-insensitive pathway is only about 0·7 % that of the aerobic oxidation of Co I H_2. In fact, although BAL treatment practically completely inactivates the aerobic oxidation, the residual aerobic oxidation, shown in curve 6 of Fig. 8, is nevertheless somewhat more rapid than the residual anaerobic oxidation measured by the differences between curves 2 and 5. It is quite possible that many reactions of this order of velocity may occur, for example diaphorase may be able slowly to react with cytochrome *b* directly. Fumarate hydrogenase, isolated by Fischer & Eysenbach (1937) from yeast, cannot be responsible for this oxidation since the BAL-insensitive reaction, as well as the BAL-sensitive reaction, was inhibited by malonate. Malonate has no effect on fumarate hydrogenase.

Since Co I H_2 has a lower potential than cytochrome *b* it must eventually completely reduce the cytochrome *b* if the two are capable of reaction. Actually complete reduction was obtained on standing, only when both cyanide was added and as much oxygen as possible was removed. In the anaerobic experiments in the absence of cyanide, the factor would be very slightly oxidized by traces of oxygen remaining. If the factor has a potential of 0·11 V., i.e. half-way between that of cytochromes *b* (−0·04 V.) and *c* (0·26 V.) and is present in the same concentration as *b*, it can be calculated that if only 1 % of the factor is in the oxidized form the maximum reduction of the cytochrome *b* will be 24 %. Similarly, a very slight cyanide-stable oxidation in the presence of oxygen and cyanide would be sufficient to prevent complete reduction of the cytochrome *b* in the aerobic experiments. In addition, under these conditions, the cytochrome *b* is slightly autoxidizable (Keilin & Hartree, 1939).

It can also be calculated that the ratio of the rate of the BAL-sensitive oxidation by fumarate to the rate of aerobic oxidation of succinate (which is completely BAL sensitive) should be 1·27 % if the factor has an oxidation-reduction potential of 0·11 V. The actual ratio found was about 1·3 %. In this calculation, the following assumptions were made: (1) in the anaerobic reaction, the reduction of oxidized cytochrome *b* by reduced factor will be the rate determining step; (2) in the aerobic oxidation no reaction is limiting, so that, at the steady state, half the cytochrome *b* and half the factor will be in the reduced form; (3) the thermodynamic equations developed for reactions in solution can be applied without alteration when the reactants are attached to the colloidal particles of the heart-muscle preparation. The first of these assumptions is very probable; there is no evidence for the second, but considerable departures from the conditions assumed do not affect the final answer appreciably. The existence of this anaerobic oxidation of Co I H_2 by fumarate, its rate and its sensitivity to BAL furnishes further evidence for the existence of the factor with the properties described in this and the previous papers (Slater, 1948, 1949*a*).

The physiological significance of the anaerobic reaction is somewhat doubtful. Its relative slowness indicates that, except possibly under some special circumstances, it cannot play a very important role quantitatively in the metabolism of the cell even under anaerobic conditions. Certainly, it cannot of itself, play any role aerobically because, under these conditions, the passage of hydrogen atoms (or electrons) to fumarate cannot increase the rate of oxidation, since they enter a 'blind alley' and have to come out again if they are to reach oxygen (cf. Ball's (1942) argument against the participation of the reaction oxalacetate ⇌ malate in the oxidation of Co I H_2 and also Potter, 1940*a*). Thus this reaction provides no support for the Szent-Gyorgyi theory of tissue respiration. In fact, heart-muscle preparation contains no fumarate and neither added fumarate nor malonate has any effect on the aerobic oxidation. Thus it must be concluded in agreement with Straub (1940*b*), Potter (1940*b*) and Potter & Albaum (1943) that the oxidation of Co I H_2 is not brought about by the Szent-Gyorgyi mechanism.

Co I H_2 *oxidase and succinic oxidase systems.* While there are important differences between the Co I H_2 oxidase and succinic oxidase systems, e.g. the latter, but not the former, is inhibited by malonate and only the latter involves cytochrome *b*, the resemblances between the two systems as they exist on the particles of the heart-muscle and kidney preparations are perhaps more striking than their differences. Thus both systems are inhibited reversibly by the powerful poisons of cell respiration such as potassium cyanide, hydrogen sulphide and azide and the inhibitions by carbon monoxide are reversed by light. Similarly, the two systems are affected by BAL in the same way. The difference in behaviour of the succinic oxidase system in heart-muscle and kidney prepara-

tions to added cytochrome *c* is repeated with the Co I H_2 oxidase system. Both systems are affected in the same way by the concentration of phosphate.

Even quantitatively the two systems have the same order of activity. The mean Q_{O_2} (μl. of oxygen/mg. of fat-free dry wt./hr.) of the Co I H_2 oxidase system, calculated from the rate of decline of the optical density at 340 mμ., the molecular extinction coefficient of Co I H_2 and the equation

$$\text{Co I } H_2 + \tfrac{1}{2}O_2 \rightarrow \text{Co I} + H_2O,$$

was found to be 131 at 20°. If the temperature coefficient found between 17·1 and 23·2° holds up to 38°, as is probable, this Q_{O_2} will be 870 at 38°. This may be compared with the average Q_{O_2} for the succinic oxidase system and of the cytochrome oxidase portion previously reported (Slater, 1949*c*), viz. 500 (in absence of added cytochrome *c*) and 3200 respectively at 38°. The corresponding figures for the kidney preparation are Co I H_2 oxidase (with excess cytochrome *c*), 40 at 20°, equivalent to 270 at 38°; succinic oxidase (with excess cytochrome *c*), 200 at 38°; cytochrome oxidase, 1380 at 38°. It will be shown in the next paper (Slater, 1950) that Co I H_2 and succinate reduce added cytochrome *c* at about the same rate in the presence of heart-muscle preparation.

The Co I H_2 and succinic oxidase systems together probably account for a considerable proportion of the total respiration of the muscle, since succinate is undoubtedly an important intermediary metabolite and several metabolites require cozymase for their oxidation.

SUMMARY

1. The method of measuring the dihydrocozymase (Co I H_2) oxidase activity of heart-muscle and kidney preparations is described.

2. Co I H_2 rapidly and completely reduced cytochromes *a*, a_3 and *c* when added to heart-muscle preparation. Cytochrome *b* was slowly and usually incompletely reduced. It was reoxidized on the subsequent addition of fumarate.

3. The Co I H_2 oxidase system was inhibited by cyanide, sulphide, azide, hydroxylamine and carbon monoxide. The inhibition by cyanide was completely reversed by dilution and that by carbon monoxide completely reversed by strong light.

4. The oxidase system was also inhibited to the extent of 99% by treatment of the heart-muscle preparation with BAL (2:3-dimercaptopropanol) in air. The Co I H_2 oxidase system was rather more susceptible than the succinic oxidase system to low concentrations of BAL. The degree of inactivation caused by BAL was not affected by the addition of diaphorase or of cytochrome *c* after the BAL treatment.

5. Added cytochrome *c* had only a slight effect on the Co I H_2 oxidase system in heart-muscle preparation, but increased the activity of kidney preparation by 11-fold.

6. When the aerobic oxidation of Co I H_2 was prevented either by removing oxygen or by adding cyanide, the Co I H_2 was completely oxidized by fumarate. This anaerobic oxidation was only one-fiftieth as rapid as the aerobic oxidation of Co I H_2 or succinate. It was inhibited by malonate and, to the extent of 60%, by treatment of the heart-muscle preparation with BAL.

7. The diaphorase activity of heart-muscle preparation (measured by extrapolation to infinite methylene blue concentration) was 3–4 times that of the Co I H_2 oxidase system (measured in the absence of added cytochrome *c*).

8. The Co I H_2 oxidase activities of heart-muscle and kidney preparations are about the same as the succinic oxidase activities at room temperature. Since, however, the temperature coefficient of the former is much higher than that of the latter, the Co I H_2 oxidase system is appreciably the more active at 38°.

9. It is suggested that the components of the Co I H_2 oxidase system are diaphorase, the BAL-sensitive factor, cytochrome *c*, cytochrome *a* and cytochrome a_3.

I wish to thank Prof. D. Keilin, F.R.S., for his interest in and advice during this investigation and the Australian National University for a Research Fellowship.

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The Dihydrocozymase-cytochrome *c* Reductase Activity of Heart-muscle Preparation

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The dihydrocozymase-cytochrome *c* reductase may be defined, following Potter & Albaum (1943), as that portion of the Co I H_2-oxidase system which is concerned with the reduction of cytochrome *c* by Co I H_2. According to the scheme proposed in the previous paper, it may be represented thus

$$\text{Co I } H_2 \rightarrow \underbrace{\text{Diaphorase} \rightarrow \text{Factor} \rightarrow \text{cytochrome } c}_{\text{Co I } H_2\text{-cytochrome } c \text{ reductase}} \rightarrow \text{cytochrome } a \rightarrow \text{cytochrome } a_3 \rightarrow O_2.$$

This paper is concerned with some properties of this enzyme complex in Keilin & Hartree's (1947) heart-muscle preparation. No attempt has been made to obtain it in solution and purify it.

METHODS

Cytochrome c *reductase activity* was measured by following the reduction of cytochrome *c* in a Beckman spectrophotometer at 550 mμ., using 1 cm. cells. The reoxidation of the cytochrome *c* was prevented by adding cyanide (0·001 M). The diluted heart-muscle preparation was added to cytochrome *c* in phosphate buffer, pH 7·3 (final concentration 0·125 M) in the Beckman cell several minutes before zero time in order completely to oxidize the cytochrome *c*. At zero time, the cyanide and Co I H_2 solutions were added and the optical density at 550 mμ. measured at approximately 20 sec. intervals. The rates of reduction of cytochrome *c* were sufficiently rapid so that only a small amount of oxidized cytochrome *c* combined with cyanide during the course of the reaction (Potter, 1941; Horecker & Kornberg, 1946). The 'blank' cuvette contained cytochrome *c*, heart-muscle preparation, cyanide and phosphate buffer. Corrections for temperature were made as described in the previous paper (Slater, 1950).

Optical densities at 550 mμ. were converted to molar concentrations of reduced cytochrome *c* by multiplying by the factor $5{\cdot}35 \times 10^{-5}$. This factor was calculated from the molar extinction coefficient at 550 mμ. of reduced cytochrome *c*, reported by Theorell & Akesson (1941), and the observation that, under the conditions of the experiments described in this paper, the optical density of oxidized cytochrome *c* at 550 mμ. was 0·34 times that of reduced cytochrome *c*.

Other measurements were made as described in the previous paper (Slater, 1950). The Co I H_2 was prepared by enzymic reduction. Optical densities at 340 mμ. were con-

verted to molar concentrations of Co I H_2 by multiplying by the factor $16{\cdot}1 \times 10^{-5}$ (calculated from Horecker & Kornberg, 1948).

EXPERIMENTS

In Fig. 1, curve *A*, the course of the reduction of cytochrome *c* by Co I H_2 in the presence of very dilute heart-muscle preparation (0·021 mg. of fat-free dry wt./ml.) is shown. This reduction follows practically the same course as that obtained when succinate was the reducing agent (curve *B*). Thus, at the temperature of this experiment (27°), the Co I H_2-cytochrome *c* reductase activity of the heart-muscle preparation was practically the same as its succinate-cytochrome *c* reductase activity. Curve *C* shows the course of the oxidation of Co I H_2 in the absence of added cytochrome *c* or cyanide.

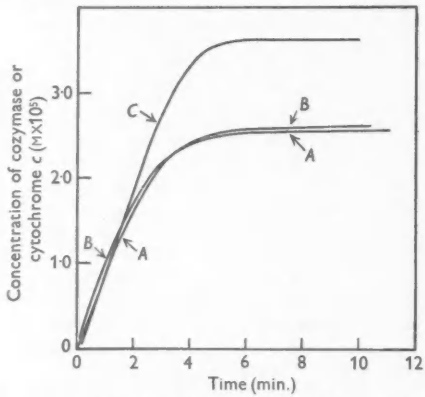

Fig. 1. Reduction of cytochrome *c* by succinate and Co I H_2 in presence of heart-muscle preparation. Phosphate buffer (pH 7·3), 0·125M; cytochrome *c*, $2{\cdot}6 \times 10^{-5}$M; Co I H_2, $3{\cdot}67 \times 10^{-5}$M; succinate, 0·0133M; KCN, 0·001M; heart-muscle preparation, 0·021 mg. of fat-free dry weight/ml. Experiments carried out at 27·0–27·5°. No correction for temperature. Curve *A*, formation of reduced cytochrome *c* by Co I H_2; curve *B*, formation of reduced cytochrome *c* by succinate; curve *C*, disappearance of Co I H_2 in absence of cyanide or cytochrome *c*.

The reduction of different concentrations of cytochrome *c* by Co I H_2 and the heart-muscle preparation is shown in Fig. 2. In Fig. 3, log. oxidized cytochrome *c* concentration at time *t* is plotted against time *t*. It is apparent that the points do not fall on a straight line, except at very low concentrations of cytochrome *c*. Thus the reduction of cytochrome *c* by Co I H_2 and heart-muscle preparation cannot be described by the simple unimolecular equation, which holds for the dihydrocoenzyme II-cytochrome *c* reductase isolated by Haas, Horecker & Hogness (1940). When the rate of reduction was plotted against the concentration of oxidized cytochrome *c*,

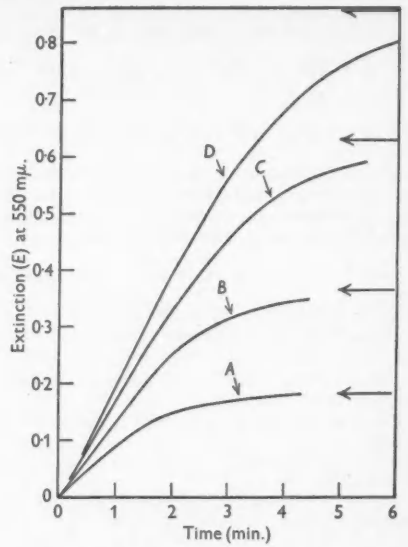

Fig. 2. Reduction of cytochrome *c* by Co I H_2 in presence of heart-muscle preparation (same preparation as in Fig. 1). Phosphate buffer (pH 7·3), 0·125M; Co I H_2, $3{\cdot}67 \times 10^{-5}$M; KCN, 0·001M; heart-muscle preparation, 0·021 mg. of fat-free dry wt./ml. No correction for temperature. Curve *A*, $1{\cdot}04 \times 10^{-5}$M cytochrome *c*, 28°; curve *B*, $2{\cdot}08 \times 10^{-5}$M cytochrome *c*, 28·1°; curve *C*, $3{\cdot}64 \times 10^{-5}$M cytochrome *c*, 28·5°; curve *D*, $5{\cdot}2 \times 10^{-5}$M cytochrome *c*, 28·8°. The horizontal arrows show the final value reached.

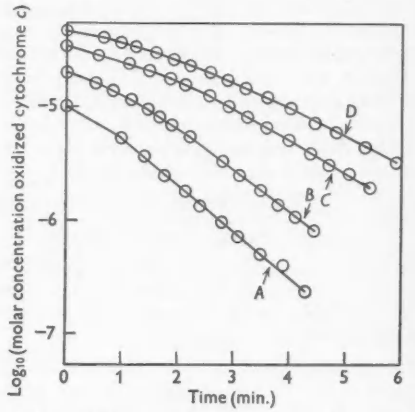

Fig. 3. Rate of decrease of the logarithm of the oxidized cytochrome *c* concentration, calculated from Fig. 2. Curve numbers as in Fig. 2.

a fairly close fit to a rectangular hyperbola was obtained. The fit was not sufficiently close, however, to allow a reliable extrapolation to infinite cytochrome

c concentration. The maximum rate of reduction of cytochrome *c* found in Fig. 2 was $0{\cdot}42 \times 10^{-5}$ mole litre^{-1}, corrected to 20°, when the initial concentration of oxidized cytochrome *c* was $5{\cdot}2 \times 10^{-5}$ M.

Using a highly purified sample of cozymase, Hogeboom (1949) found that the amounts of Co I H_2 oxidized and cytochrome *c* reduced when both were added to a preparation from rat liver agreed with the equation

$$\text{Co I } H_2 + 2c^{\cdot\cdot\cdot} \rightarrow \text{Co I} + 2c^{\cdot\cdot} + 2H^{\cdot},$$

where $c^{\cdot\cdot\cdot}$ and $c^{\cdot\cdot}$ are oxidized and reduced cytochrome *c* respectively. With the sample of cozymase used in the present investigation, there was good agreement with this equation when an excess of cytochrome *c* was added to the Co I H_2. When, however, the Co I H_2 was in excess, as in Figs. 1 and 2, about 25% more cytochrome *c* was reduced than could be accounted for by the amount of Co I H_2 oxidized, probably due to the presence in the cozymase preparation of impurities capable of reducing cytochrome *c*. The reduction of cytochrome *c* by these impurities does not affect any of the conclusions drawn in this paper.

Applying the above equation, the initial rate of oxidation of Co I H_2 by heart-muscle preparation and $5{\cdot}2 \times 10^{-5}$ M-cytochrome *c* would be $0{\cdot}21 \times 10^{-5}$ mole litre^{-1} min.$^{-1}$ at 20°. This may be compared with the initial rate of oxidation in the absence of added cytochrome *c* or cyanide calculated from curve *C*, Fig. 1, viz. $0{\cdot}40 \times 10^{-5}$ mole litre^{-1} min.$^{-1}$. Thus, even this very high concentration of added cytochrome *c* is not as active as the heart muscle alone in bringing about the oxidation of Co I H_2.

The relative rate of reduction of the endogenous cytochrome *c* of the preparation compared with that of added cytochrome *c* was calculated in the following way. It was found from Fig. 3 that at low concentrations of cytochrome *c*, the rate of reduction at 20° was expressed by the equation $-dc/dt = 0{\cdot}39c$, where *c* is the concentration of added oxidized cytochrome *c*. The concentration of the endogenous cytochrome *c* of the heart-muscle preparation at the dilution employed was calculated from the fat-free dry weight content of the preparation and the figures previously given (Slater, 1949*a*) to be $1{\cdot}7 \times 10^{-8}$ M. If this endogenous cytochrome *c* were reduced by the Co I H_2 at the same rate as added cytochrome *c*, the rate of reduction would be $0{\cdot}39 \times 1{\cdot}7 \times 10^{-8} = 0{\cdot}666 \times 10^{-8}$ mole litre^{-1} min.$^{-1}$. The rate of reduction of the cytochrome *c*, calculated from the observed rate of disappearance of the Co I H_2 in the absence of cyanide or added cytochrome *c*, was $2 \times 0{\cdot}40 \times 10^{-5} = 0{\cdot}8 \times 10^{-5}$ mole litre^{-1} min.$^{-1}$. The rate of reduction of the endogenous cytochrome *c* is then 1200 times that of added cytochrome *c*. Thus the endogenous cytochrome *c* of heart-muscle preparation is about a thousand times more active than added cytochrome *c* in catalysing the oxidation of Co I H_2. Similarly, it is much more active in the oxidation of succinate and *p*-phenylene-diamine (Slater, 1949*a*, *b*).

The relation $-dc/dt = 0{\cdot}39c$ enables a calculation of the 'specific cytochrome reductase activity' of the heart-muscle preparation in the units of Haas *et al.* (1940). This value was 13·7 at 25°.

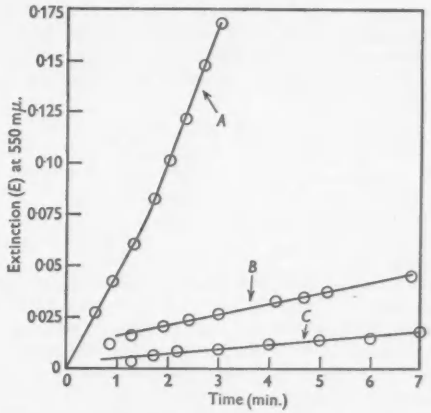

Fig. 4. Effect of BAL treatment on Co I H_2-cytochrome *c* reductase activity of heart-muscle preparation. Phosphate buffer (pH 7·3), 0·125 M; Co I H_2, 6×10^{-5} M; KCN, 0·001 M; heart-muscle preparation, 0·020 mg. of fat-free dry wt./ml. Temperature 24·0–24·8°. No correction for temperature. Curve *A*, heart-muscle preparation shaken in air for 15 min. at 37° before dilution; curve *B*, heart-muscle preparation shaken in air with 0·01 M-BAL for 15 min. at 37° before dilution; curve *C*, reduction in the absence of heart-muscle preparation.

Treatment of the heart-muscle preparation with 2:3-dimercaptopropanol (BAL) strongly inactivated its Co I H_2-cytochrome *c* reductase (Fig. 4). When the rates of reduction were corrected for the small reduction by the Co I H_2 preparation alone (curve *C*), it was found that the BAL treatment (0·01 M-BAL for 15 min. at 37°) had inactivated the Co I H_2-cytochrome *c* reductase by 94·6%. The inactivation of the Co I H_2 oxidase was not measured at the same time, but from other experiments with heart-muscle preparations of similar concentration, it would be expected that the inactivation would be about 90–95%. The initial lag in the reduction of cytochrome *c* by the control heart-muscle preparation parallels the lag of the oxidation of Co I H_2 in the absence of cyanide or added cytochrome *c* after shaking heart-muscle preparation at 37° for 15 min. (Fig. 4; Slater, 1950). Although there was some unoxidized BAL left in the heart-muscle preparation after treatment with BAL, the final concentration of this BAL after dilution of the heart-muscle prepara-

tion was so low that it did not cause any reduction of the cytochrome *c* before the addition of the Co I H_2.

DISCUSSION

It was concluded in the previous paper that a factor, or factors, links diaphorase with cytochrome *c*. This factor, or one of them, is the BAL-sensitive factor, which also mediates the oxidation of cytochrome *b* by cytochrome *c* in the succinic oxidase system. According to this conclusion, the reaction between Co I H_2 and cytochrome *c* may be described by the equations

$$(1)\quad \text{Co I } H_2 + D \rightarrow \text{Co I} + DH_2,$$
$$(2)\quad DH_2 + 2F^{\cdot\cdot\cdot} \rightarrow D + 2F^{\cdot\cdot} + 2H^{\cdot},$$
$$(3)\quad 2F^{\cdot\cdot} + 2c^{\cdot\cdot\cdot} \rightarrow 2F^{\cdot\cdot\cdot} + 2c^{\cdot\cdot},$$

overall reaction

$$\text{Co I } H_2 + 2c^{\cdot\cdot\cdot} \rightarrow \text{Co I} + 2c^{\cdot\cdot} + 2H^{\cdot},$$

where *D* and DH_2 are oxidized and reduced diaphorase, $F^{\cdot\cdot\cdot}$ and $F^{\cdot\cdot}$ are oxidized and reduced factor (assumed, for purposes of these equations, to be a haematin compound), $c^{\cdot\cdot\cdot}$ and $c^{\cdot\cdot}$ are oxidized and reduced cytochrome *c*. Each of these equations probably represents a number of reactions.

It has been shown in the previous paper that reaction (1) is more rapid than the overall reaction. The fact, also reported in the previous paper, that added cytochrome *c* has little stimulating effect on the aerobic oxidation of Co I H_2 suggests that reaction (3) is also not limiting when the $c^{\cdot\cdot}$ formed by reaction (3) is rapidly reoxidized by cytochrome *c* oxidase. Thus it seems likely that, in the absence of cyanide, reaction (2) is the slowest reaction of the series. In the presence of cyanide, however, the oxidation of $c^{\cdot\cdot}$ is prevented, owing to inhibition of the cytochrome oxidase, so that when the small amount of endogenous cytochrome *c* is reduced, it is removed from the reaction. Reaction (3) can now be restored by adding a large excess of cytochrome *c*, but added cytochrome *c* reacts with the factor very much more slowly than does the endogenous cytochrome *c* of the heart-muscle preparation. Neither added nor endogenous cytochrome *c* reacts directly with diaphorase, since treatment of the heart-muscle preparation with BAL inactivates the aerobic oxidation of Co I H_2 and the Co I H_2-cytochrome *c* reductase without affecting the reaction between Co I H_2 and methylene blue. (There might be a very slow direct reaction between the endogenous cytochrome *c* and diaphorase, since treatment of the heart-muscle preparation with BAL under such conditions that the succinic oxidase activity is completely inactivated, does not completely stop the aerobic oxidation of Co I H_2. The residual oxidation usually amounts to 1–2% that of the untreated preparation.)

The conclusion that at least two enzymes are required for the reduction of cytochrome *c* by Co I H_2 might appear at variance with the findings of several workers who have obtained various cytochrome reductases in solution. There are, however, several possible explanations of this apparent discrepancy.

(i) In no case is there unequivocal evidence that only one enzyme was concerned in the reaction between the coenzyme and cytochrome *c*, under the conditions of test used. The Co I H_2-cytochrome *c* reductase of animal tissues recently obtained in solution by Heppel (1949) and that extracted from yeast by Altschul, Persky & Hogness (1941) have not been completely purified. In this connexion, it is interesting to note that crude solutions of yeast lactic dehydrogenase catalyse the reduction of cytochrome *c* by lactate, but this property is lost on purification, presumably due to the removal of an additional factor (Bach, Dixon & Zerfas, 1946). Dihydrocoenzyme II-cytochrome *c* reductase has been isolated from yeast and highly purified by Haas *et al.* (1940) and Haas, Harrer & Hogness (1942) (see also Ochoa, 1945). However, although as judged by flavine content and other measurements this enzyme was very pure, considerable amounts of crude enzyme preparations have been added in the tests for cytochrome reductase activity which have been published. (I am indebted to Dr C. Liébecq for drawing my attention to this.) It is possible that these crude enzyme preparations provided an additional factor. Horecker (1949) has recently briefly reported the isolation of dihydrocoenzyme II-cytochrome *c* reductase from animal tissues. The specific activity of this enzyme is one-sixth that of the yeast enzyme, i.e. 158/6 = 26, which may be compared with 13·7 for the Co I H_2-cytochrome *c* reductase activity of unpurified heart-muscle preparation found in the present study.

(ii) Diaphorase II, which catalyses the oxidation of dihydrocoenzyme II by methylene blue (Adler, Euler & Günther, 1939) may react directly with cytochrome *c*, although diaphorase I requires an additional factor.

(iii) Different enzyme systems may be involved in yeast and animal tissues.

(iv) Even if an enzyme in solution rapidly reduces a large concentration of cytochrome *c*, the two enzymes may not interact directly when they are attached to granules in the cell. (The converse is equally possible since two enzymes which interact very rapidly *in vivo* when attached to granules may react much more slowly when the two are in solution.) The enormously greater rate of reduction of the endogenous cytochrome *c* of the heart-muscle preparation compared with that of added cytochrome *c* is an illustration of the differences between the two forms of cytochrome *c*. Moreover, cyto-

chrome *c* in solution is reduced very rapidly by a number of reducing agents, e.g. ascorbic acid which reduce the cytochrome *c* in the heart-muscle preparation much more slowly (Slater, 1949*b*). Thus the various 'cytochrome reductases' which have been isolated may be the appropriate diaphorase, which when reduced, can be reoxidized either by methylene blue or cytochrome *c* in solution, but which *in vivo* is not oxidized by cytochrome *c* but by another catalyst, which may be a haematin compound like cytochrome *c* (Slater, 1949*c*).

SUMMARY

1. The Co I H_2-cytochrome *c* reductase activity of heart-muscle preparation is about the same as its succinate-cytochrome *c* reductase activity at room temperature.

2. The concentration of oxidized cytochrome *c* does not fall exponentially with time during the course of the reduction. The rate of reduction of cytochrome *c* is approximately a rectangular hyperbolic function of the oxidized cytochrome *c* concentration.

3. The rate of reduction of the endogenous cytochrome *c* of the heart-muscle preparation, calculated from the rate of the aerobic oxidation of Co I H_2, was 1200 times that of the same concentration of cytochrome *c* added to the heart-muscle preparation.

4. The Co I H_2-cytochrome *c* reductase activity of heart-muscle preparation is inactivated by treatment with BAL.

5. It is concluded that the Co I H_2-cytochrome *c* reductase in heart-muscle preparation consists of two or more enzymes.

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On Certain Peptides Occurring in Marine Algae

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In an earlier communication Haas, Hill & Russell-Wells (1938) expressed their intention of investigating the water-soluble peptide occurring in the red alga *Griffithsia flosculosa* Batt. (*G. setacea* C. Ag.). The outbreak of war, however, interrupted the work, and further collaboration became impossible. The present communication is an attempt to amplify the earlier work on *G. flosculosa*, and to provide certain additional data concerning the peptides contained in two species each of the brown alga *Pelvetia* and the encrusted alga *Corallina*, belonging to the Phaeophyceae and Rhodophyceae respectively.

METHODS

The freshly gathered weed was spread out to dry and worked up as required, drying having been found to have no effect on the nature of the extract. For extraction the dried weed was immersed in 4–5 times its weight of water and extracted for about 1 hr. at 60–70° with occasional stirring; after straining the weed, a second extraction was made and the combined extracts were filtered through paper pulp and precipitated by basic Pb acetate; the filtrate from this was freed from Pb by saturation with H_2S and the resultant acid solution was concentrated under reduced pressure at about 60°. The resulting solution was made alkaline with Na_2CO_3, freed from NH_3 by a rapid current of air, and after neutral-

izing was precipitated by mercuric acetate with alternate addition of Na_2CO_3 to neutralize any acidity. The precipitated material was repeatedly washed by decantation and centrifuging and finally decomposed by H_2S. The resulting filtrate was evaporated to dryness under reduced pressure.

RESULTS

The peptide of Griffithsia flosculosa

It was previously suggested (Haas & Hill, 1933) that the occurrence of peptides of this kind might be due to restricted photosynthesis resulting in the production of insufficient carbohydrate for normal metabolism of intermediate products in the synthesis and breakdown of protein. Such conditions would seem to apply to *Griffithsia*, which normally grows in shaded pools. At Parke's Beach, Lymington, Hants, from which locality practically all the material for examination was collected, it was growing below the *Laminaria* zone and was only exposed to full daylight for a couple of hours at fortnightly intervals of low spring tides, a circumstance which considerably restricted opportunities for collecting material.

Records of the occurrence of the peptide, as indicated by the biuret reaction, are given below:

	March	April	July	August	September	December
Biuret reaction	+	+	Faint	Absent	Absent	+

It will be observed that the biuret reaction is either faint or absent during the summer months,* an observation which lends some support to the suggestion that lack of light may be a factor contributing to the occurrence of the peptide at other times of the year.

Tests for certain constituent amino-acids in the samples of peptide extracted at the various seasons revealed no significant difference in composition throughout the year; in all cases the same four—glycine, alanine, arginine and histidine—were found.

It must therefore be concluded that the peptide is not a constant constituent of *Griffithsia flosculosa*, but that when it does occur its qualitative composition does not appear to vary, at any rate in so far as the four simple amino-acids are concerned; there is, however, no evidence to show whether it is an upgrade or downgrade product of metabolism.

* A specimen of *Griffithsia* collected in September in an entirely different locality, namely Poole Harbour, likewise contained no peptide.

It is fully recognized that the interpretation of the results here presented requires considerably more evidence than is offered in this communication, but lack of opportunity for securing further evidence by the collection of other similarly situated algae and the length of time required for studying seasonal variation prevents continuation of the investigation and for this reason the observations so far made are published despite their incompleteness in the hope that they may be of interest to future investigators.

The peptides of Pelvetia canaliculata, Pelvetia canaliculata *forma* libera, Corallina officinalis *and* Corallina squamata

The above algae, though belonging to different families and growing under entirely different conditions, share the common characteristic of containing peptides. In the case of the two species of *Pelvetia*, reduced photosynthesis could be accounted for by prolonged periods of intertidal desiccation which, on the salt marsh at Blakeney Point, extends to about a fortnight between spring tides for the free floating *P. canaliculata* forma *libera*.

The occurrence of peptides in calcareous algae such as the two species of *Corallina* could again be attributed to reduced photosynthesis due to restricted penetration of light through the encrusting calcareous sheath.

No systematic attempt was made to investigate the question of seasonal variation of peptide in these weeds, the results recorded applying only to isolated examples.

Pelvetia canaliculata was collected in Plymouth and *P. canaliculata* f. *libera* was collected at Blakeney Point, Norfolk.

Of the two species of calcareous algae *Corallina officinalis* was obtained from Plymouth in February 1948, and *C. squamata* was gathered at Dancing Ledge, Dorset, in May 1948. This latter sample was found to be free from peptide, though material collected from the same locality at other periods of the year gave the biuret reaction, thus suggesting that this weed is likewise subject to seasonal variation.

Table 1 shows the qualitative composition of the peptides examined. Tyrosine and tryptophan were notably absent from the peptides in all cases.

Table 1. *Qualitative composition of peptides from marine algae*

	Glycine	Alanine	Arginine	Histidine	Aspartic acid	Glutamic acid
Griffithsia flosculosa	+	+	+	+	−	−
Pelvetia canaliculata	+	+	+	+	−	+
P. canaliculata f. *libera*	+	+	+	+	−	+
Corallina officinalis	+	+	+	+	+	−

Properties of the peptides

The various peptides were very similar in appearance, being pale-yellow or light-brown semi-solids, which when quite dry were almost brittle like resin, but were deliquescent and readily soluble in water and diffusible through a cellophan membrane. They were not precipitated by basic lead acetate, copper sulphate, picric, tannic or trichloroacetic acids, but they were precipitated by mercury salts. The ordinary colour reactions for arginine, histidine, tyrosine and tryptophan were carried out on the intact peptide, but for the detection of alanine and glycine an adaptation of methods suggested by Alexander & Seligman (1945) and Alexander, Landwehr & Seligman (1945) were employed on the hydrolysed product prepared by heating the peptide for some hours with 20% (w/v) aqueous hydrochloric acid and evaporating to dryness; the method employed depends upon the reaction of the amino-acids with ninhydrin and the identification by colour tests of the formaldehyde or acetaldehyde produced from glycine or alanine respectively.

To this end 5 ml. of 0·1% (w/v) solution of the hydrolysate was distilled with 2 ml. of phosphate buffer pH 5·5, 1 ml. of 1% (w/v) aqueous ninhydrin and a glass bead, in a short-necked round-bottomed flask; the latter was connected to a 4 mm. diam. condenser tube bent to reach the bottom of a 10 ml. graduated cylinder, containing 2 ml. of 1% (w/v) aqueous $NaHSO_3$ standing in ice water. The liquid was distilled briskly so that about 7 ml. were collected in as many minutes and the distillate was tested as follows.

Glycine. To 5 ml. were added in portions 4 ml. of conc. H_2SO_4 with repeated shaking and cooling. When cold, 3 drops of 5% (w/v) aqueous chromotropic acid were added and the mixture was warmed in a boiling water bath. If glycine is present a deep red-violet colour develops almost at once and increases in depth as heating is continued.

Alanine. 1 ml. of the ice-cold distillate was carefully poured into 8 ml. of conc. H_2SO_4 cooled in ice. One drop of 5% (w/v) aqueous $CuSO_4$ and 0·2 ml. of *p*-hydroxydiphenyl (1·5% (w/v) in 0·12N-NaOH) were then added. With alanine, an immediate white precipitate results which, on vigorous shaking, gives way to a clear blue colour. This can be intensified by immersing in a boiling water bath for a minute or two.

The two colour reactions do not interfere with each other, and the same solution can be used for the detection of either or both of the amino-acids concerned.

DISCUSSION

The examination of certain water-soluble peptides extracted from a small selection of marine algae leads to the conclusion that these substances are not of constant occurrence throughout the year. While the observations here recorded are hardly sufficient to justify drawing any definite conclusions, it may at least be said that they are not at variance with the hypothesis that the peptides may be intermediate products of protein metabolism resulting from deficient photosynthetic activity caused by unfavourable conditions of growth, either as regards illumination or moisture.

In view of the possibility of these peptides having antibiotic properties, samples were submitted for testing against various bacteria, but only the peptide from *Corallina officinalis* exhibited any activity in a concentration of 1 mg./ml. at which it inhibited *Streptococcus pyogenes* Richards. In view of this no detailed or quantitative analysis of these peptides was undertaken.

SUMMARY

1. Evidence is supplied in support of the suggestion that the occurrence of water-soluble peptides in *Griffithsia flosculosa* and *Corallina squamata* is subject to seasonal variation.

2. The isolation and properties of such peptides are described.

3. The peptide obtained from *Corallina officinalis* alone showed any antibiotic activity.

The author wishes to record his indebtedness to his former colleagues Prof. T. G. Hill and Dr Barbara Russell-Wells for their collaboration.

Thanks are also due to Dr Phyllis Sanderson for help in the preparation of a sample of one of the peptides, to Dr M. Park for a supply of *Corallina officinalis* and *Pelvetia canaliculata* both from Plymouth, and to Drs P. d'Arcy Hart, T. S. Work, A. T. Fuller and D. Rowley for testing the activity of the peptides against a variety of bacteria.

Note added 21 *March* 1950. Dekker, C. A., Stone, D. & Fruton, J. S. (1949, *J. biol. Chem.* **181**, 719) have recently established the composition of the peptide contained in *Pelvetia fastigiata*, a closely related species of *P. canaliculata*.

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The Constitution of Hydroxysulphapyridine and Synthesis of 2-Aminohydroxypyridines

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Hydroxysulphapyridine was isolated as a metabolite of sulphapyridine in the dog by Scudi (1940, 1944) and by Weber, Lalich & Major (1943). In an investigation of the metabolism of sulphapyridine in the rabbit, we obtained a compound which appeared to be identical with the hydroxysulphapyridine produced by the dog (Bray, Neale & Thorpe, 1946). The constitution was not determined, but evidence was obtained that one hydroxyl group had been introduced. Scudi (1944) had suggested that this occupied either position 3 in the pyridine ring or position 2 in the benzene ring. Since, however, hydrolysis of hydroxysulphapyridine gave sulphanilic acid and a compound believed to be an aminohydroxypyridine it was more reasonable to infer that the hydroxyl was attached to the pyridine ring (Weber *et al.* 1943; Bray *et al.* 1946). In the expectation that hydroxylation might occur in position 5, we synthesized a compound which the literature led us to believe was 2-amino-5-hydroxypyridine. Since this compound was different from that obtained from the rabbit we attempted to synthesize the other three isomers. This investigation revealed that the so-called '2:5'-compound was really the 2:3-isomer. Of the four possible isomers two, 2-amino-3-hydroxypyridine and 2-amino-6-hydroxypyridine, have been obtained by routes which leave little doubt of their configuration. We have not succeeded in preparing 2-amino-5-hydroxypyridine, although we have obtained strong evidence that it does indeed form the aminohydroxypyridine moiety of hydroxysulphapyridine. Since we met with considerable difficulty in the preparation of aminohydroxypyridines and found some discrepancies in the literature, we include in this report a brief summary of some of the syntheses carried out.

EXPERIMENTAL

Synthesis of aminohydroxypyridines

2-Amino-3-hydroxypyridine was prepared by three separate methods.

Method 1. 8-Hydroxyquinoline was oxidized with fuming HNO_3 to yield quinolinic acid, m.p. 190° (decomp.), from which quinolinimide was obtained by treatment with acetic anhydride and acetamide (Sucharda, 1925). The imide, with NaClO, gave 3-aminopyridine-2-carboxylic acid (3-aminopicolinic acid), m.p. 214°, and 2-aminopyridine-3-carboxylic acid (2-aminonicotinic acid) which were separated, the latter being less soluble in acid solution. 3-Aminopicolinic acid was converted to 3-hydroxypicolinic acid, m.p. 218–220°, by decomposition of the diazonium salt (Kirpal, 1908) and thence via the methyl ester (Camps, 1902) to *3-hydroxypicolinamide* which formed colourless needles, m.p. 194°, and gave a red colour with $FeCl_3$. (Found: N, 20·2; $C_6H_6O_2N_2$ requires N, 20·3 %.) This amide on treatment with NaBrO gave 2-amino-3-hydroxypyridine, m.p. 169–170°, Plazek & Rodewald (1936) give m.p. 164–168°. (Found: N, 25·2. Calc. for $C_5H_6ON_2$: N, 25·5 %.) Yield, 2 g. from 50 g. of 8-hydroxyquinoline. 2-Amino-3-hydroxypyridine gave a purple coloration with $FeCl_3$, a negative diazo reaction, and no colour with diazotized 2:5-diaminopyridine in alkaline ($NaHCO_3$) solution. It formed a picrate, m.p. 257° (decomp.).

Method 2. Pyridine was sulphonated with fuming H_2SO_4 ($2HgO.HgSO_4$ catalyst) (cf. Wulff, 1932*a*), and the resulting 3-sulphonate was fused with KOH to give 3-hydroxypyridine, m.p. 127–129° (cf. Wulff, 1932*b*). This was nitrated (I. G. Farbenindustrie, 1933). According to Maier-Bode & Altpeter (1934) the product, m.p. 68–69°, is 6-nitro-3-hydroxypyridine. Plazek & Rodewald (1936), however, state that the product is 2-nitro-3-hydroxypyridine. We concur, since our product, m.p. 67–68°, on reduction with $SnCl_2$ and HCl gave 2-amino-3-hydroxypyridine, identical with that prepared by Method 1. Yield, 5 g. from 50 g. of 3-hydroxypyridine (from 133 g. of pyridine).

Method 3. Pyridine was brominated (Englert & McElvain, 1929) and the resulting 3-bromopyridine (b.p. 168–172°) treated with Na ethoxide (Weidel & Blau, 1885). The product, 3-ethoxypyridine (b.p. 190–192°), on nitration gave 2-nitro-3-ethoxypyridine (m.p. 31–32°). This was reduced to 2-amino-3-ethoxypyridine (m.p. 80–81°), which was de-ethylated by means of HBr and acetic acid at 130° to give 2-amino-3-hydroxypyridine, identical with that given by Methods 1 and 2. Yield, 5 g. from 50 g. of 3-bromopyridine (from 250 g. of pyridine). The procedure in the later stages was essentially that of Koenigs, Gerdes & Sirot (1928). It will be evident from our later experiments that 2-amino-5-hydroxypyridine may be formed in traces as a by-product in this synthesis.

2-Amino-4-hydroxypyridine. Attempts at the synthesis of this compound by the route picolinic acid → 4-chloropicolinic acid → 4-aminopicolinic acid → 4-hydroxypicolinic acid and thence by Hofmann degradation of the amide to 2-amino-4-hydroxypyridine were unsuccessful. In view of the evidence for the identity of the biosynthetic aminohydroxypyridine as the 2:5-isomer further syntheses of the 2:4-isomer were not attempted.

2-Amino-5-hydroxypyridine. Numerous unsuccessful attempts were made to prepare this compound. Two of these are referred to under Methods 2 and 3 for 2-amino-3-hydroxypyridine. Further methods tried included (*a*) via the benzene-azo compound from 3-hydroxypyridine and benzene

diazonium chloride, (*b*) from 2-amino-5-chloro-(or-bromo-)-pyridine, (*c*) from 5-hydroxy-α-picoline, (*d*) from 2-amino-pyridine-5-sulphonic acid, (*e*) from 2:5-diaminopyridine, and (*f*) from 2-chloro-5-nitropyridine. Theoretically it would be expected that nitration of 3-ethoxypyridine which as already shown (Method 3 above) gives 2-nitro-3-eth-oxypyridine, would result in at least some nitration in the para position. The preparation of 2-amino-3-hydroxypyri-dine by Method 3 was accordingly repeated but without any purification or recrystallization at any stage. The crude aminohydroxypyridine hydrobromide thus obtained was dissolved in absolute ethanol and the solution poured into an excess of ether, from which the solid hydrobromide crystallized. This was separated and dissolved in a small volume of water. The solution, after neutralization with $NaHCO_3$, was continuously extracted with ether to give fractions *A* (ether-soluble material) and *B* (residual liquid). The original ethereal mother liquors were evaporated to give a small amount of syrup (fraction *C*). The composition of these fractions was investigated by means of paper chromatography using a mixture of petroleum, b.p. 90–100° (1 vol.), *n*-butanol (2 vol.) and water (3 vol.) as developing solvent and $FeCl_3$ for detection. (For apparatus and pro-cedure see Bray, Thorpe & White, 1950.) Compounds with the following R_F values and $FeCl_3$ colours (in parentheses) were obtained: fraction *A*, 0·9 (brown) and 0·68 (purple); fraction *B*, 0·68 (purple) and 0·22 (purple); fraction *C*, 0·68 (purple) in very small amount.

The compound with R_F value 0·68 was shown to be 2-amino-3-hydroxypyridine, the major product of the re-action. The faster moving compound has not been identified, but the compound with R_F value 0·22 appeared to have properties identical with those of the aminohydroxypyridine from hydroxysulphapyridine. As will be seen later, there is good reason to believe this compound is 2-amino-5-hydroxy-pyridine. The amounts formed, however, were so small as to leave little hope of isolation of the compound by this method.

2-Amino-6-hydroxypyridine. This was prepared from saccharic acid which, on treatment with HBr, gave de-hydromucic acid (Phelps & Hale, 1901). This on reduction with Na amalgam gave dihydrofuran-2:5-dicarboxylic acid (Hill & Wheeler, 1901), which was converted to 2-hydroxy-pyridine-6-carboxylic acid (6-hydroxypicolinic acid), m.p. 275–278° (decomp.), by autoclaving with NH_4Br at 160–170° (Fischer, Hess & Stahlschmidt, 1912). The *methyl ester* formed white needles, m.p. 80°. (Found: N, 9·1; $C_7H_7O_3N$ requires N, 9·2%.) It was converted to the *amide*, white needles, m.p. 250°, which gave a bright orange colour with $FeCl_3$. (Found: N, 20·1; $C_6H_6O_2N$ requires N, 20·3%.) Yield, 1·1 g. from 50 g. of saccharic acid. Treatment of the amide with NaBrO gave a poor yield of 2-amino-6-hydroxy-pyridine (m.p. 198–200°). This was identical with the base prepared from a specimen of 2-amino-6-hydroxypyridine sulphate kindly given to us by Dr A. J. Ewins. The com-pound formed a picrate, m.p. 191° (decomp.), gave a purple coloration with $FeCl_3$ and a bright orange-yellow colour with diazotized 2:5-diaminopyridine in alkaline solution.

RESULTS AND DISCUSSION

Comparison of synthetic aminohydroxypyridines with that from hydroxysulphapyridine

Some properties of the aminohydroxypyridines ob-tained and those of the compound from hydroxy-sulphapyridine are recorded in Table 1. The R_F values were determined using the mixture of petroleum, b.p. 90–100°, *n*-butanol and water already mentioned. In addition to the amino-hydroxypyridines there is another theoretically possible oxidation product which would result from addition of one atom of oxygen to 2-aminopyridine. Some properties of this compound, 2-aminopyridine-1-oxide (2-amino-1-hydroxy-1:2-dihydropyridine), which has recently been prepared by Newbold & Spring (1949) are also included in Table 1. It is clear that the compound from hydroxysulphapyridine is not 2-amino-3-hydroxypyridine, 2-amino-6-hydroxy-pyridine or 2-aminopyridine-1-oxide. The negative dichloroquinonechloroimide test would be expected if the hydroxyl were in position 5 since this reagent does not usually give a blue colour with phenols in which the position para to the hydroxyl is substi-tuted (Gibbs, 1927). Scudi's (1944) choice of position 3 as the point of hydroxylation was based upon the observation that hydroxysulphapyridine gave a positive test. We confirm that hydroxysulphapyri-dine gives a colour with the reagent, but the amino-hydroxypyridine obtained from it by hydrolysis does not. In our opinion the colour given by hydroxy-

Table 1. *Comparison of aminohydroxypyridines*

	M.p. (°)	M.p. of picrate (°)	Colour with $FeCl_3$	Dichloro-quinonechloro-imide test	Colour on coupling with diazotized 2:5-diamino-pyridine	R_F value (petroleum-butanol)
2-Amino-3-hydroxypyridine	169–170	257	Purple	Deep blue	None	0·68
2-Amino-6-hydroxypyridine	198–200	191	Purple	Deep blue	Orange	0·63
2-Amino-?-hydroxypyridine from hydroxysulphapyridine	34	218	Purple	Colourless	Red	0·22
?-Amino-5-hydroxypyridine from Method 3	—	—	Purple	Colourless	Red	0·22
2-Aminopyridine-1-oxide* (2-imino-1-hydroxy-1:2-dihydropyridine)	161	Unstable	Intense pure blue	—	—	—

* Properties given by Newbold & Spring (1949).

sulphapyridine is not a typical indophenol colour. It is comparatively feeble in intensity, and the blue colour soon turns purple.

The similarity in the properties of the aminohydroxypyridine from hydroxysulphapyridine and that formed in traces by Method 3 provides evidence that these compounds are in fact 2-amino-5-hydroxypyridine. The only possible isomers which can be formed both by hydroxylation of 2-aminopyridine and by amination (via nitration) of 3-ethoxypyridine are 2-amino-3-hydroxypyridine and 2-amino-5-hydroxypyridine. The compound formed in the rabbit and as a trace product from 3-ethoxypyridine is certainly not 2-amino-3-hydroxypyridine. It is highly probable, therefore, that the pyridine moiety of hydroxysulphapyridine is 2-amino-5-hydroxypyridine and that the constitution of hydroxysulphapyridine is 2-(*p*-aminobenzenesulphonamido)-5-hydroxypyridine.

The configuration of the aminohydroxypyridine of Koenigs, Gerdes & Sirot (1928)

The three syntheses of 2-amino-3-hydroxypyridine render untenable the contention of Koenigs *et al.* (1928) and of Maier-Bode & Altpeter (1934) that 2-amino-5-hydroxypyridine was prepared from the mononitro derivatives of 3-ethoxy- or 3-hydroxypyridine. The evidence of Koenigs *et al.* for the position of the nitro group introduced into 3-ethoxypyridine was the conversion of the resulting aminohydroxypyridine into a dihydroxypyridine which was identical with that prepared by Kudernatsch (1897) by fusion of 3-hydroxypyridine with sodium hydroxide. Kudernatsch's evidence for the 2:5 configuration of this compound was that it did not give the reactions expected of 2:3-dihydroxypyridine, which was then unknown. Plazek & Rodewald (1936) maintained that nitration of 3-hydroxypyridine resulted in the entry of the nitro group into position 2 and not 6, as stated by Maier-Bode & Altpeter (1934), since the aminohydroxypyridine obtained by reduction formed a phenoxazine derivative with picryl chloride. The compounds which we obtained by the three methods described in the Experimental section were identical. The synthesis from quinolinic acid, and the fact that Methods 2 and 3 involved nitration of pyridines substituted in position 3, leave no doubt that this compound is 2-amino-3-hydroxypyridine. The claims that Methods 2 and 3 lead mainly to 2-amino-5-hydroxypyridine cannot, therefore, be accepted. The compounds derived from the mononitro derivatives of 3-ethoxy- and 3-hydroxypyridine to which Koenigs *et al.* (1928) and Maier-Bode & Altpeter (1934) attributed the 2:5 configuration were actually 2:3 substituted pyridines.

SUMMARY

1. An attempt has been made to identify the aminohydroxypyridine moiety of hydroxysulphapyridine, the metabolite of sulphapyridine in the rabbit.

2. 2-Amino-6-hydroxy- and 2-amino-3-hydroxypyridines have been synthesized and shown to differ from the biosynthetic compound.

3. Unsuccessful attempts were made to prepare 2-amino-4-hydroxy- and 2-amino-5-hydroxy-pyridine, although evidence was obtained that the latter is a minor product in the preparation of the 2:3-isomer. Claims by earlier workers to have prepared the 2:5-isomer were shown to refer to the 2:3-isomer.

4. Evidence has been obtained that the biosynthetic product is 2-amino-5-hydroxypyridine and hence hydroxysulphapyridine is 2-(*p*-aminobenzenesulphonamido)-5-hydroxypyridine.

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